PARVOVIRUS B19 INFECTION OF HEPATOCYTES AND INDUCTION OF APOPTOSIS

A Thesis in
Integrated Biosciences
by
Brian Douglas Poole

© 2004 Brian Douglas Poole

Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

August 2004
The thesis of Brian Douglas Poole was reviewed and approved* by the following:

Stanley J. Naides  
Thomas B. Hallowell Professor of Medicine; Professor of Microbiology and Immunology, and Pharmacology  
Thesis Advisor  
Chair of Committee

Robert H. Bonneau  
Associate Professor of Microbiology and Immunology

Sarah K. Bronson  
Assistant Professor of Cellular and Molecular Physiology

Michael J. Chorney  
Associate Professor of Microbiology and Immunology, and Pediatrics

Mark Kester  
Professor of Pharmacology

Anita K. Hopper  
Professor of Biochemistry and Molecular Biology  
Co-Director, Integrative Biosciences Program

*Signatures are on file in the Graduate School
ABSTRACT

Parvovirus B19 persists in multiple tissues and has been implicated in a variety of diseases including acute fulminant liver failure (AFLF). Despite multiple reports demonstrating the presence of B19 DNA in liver tissues from patients with AFLF, the causal relationship of B19 infection to liver failure is not yet proven. In addition, the mechanism by which B19 infection induces liver failure remains unknown. Although hepatocytes express the B19 receptor, globoside, these cells are non-permissive for B19 replication. B19, although unable to replicate productively in liver cells, may establish a limited infection that kills these cells. B19 cytotoxicity is likely mediated through the viral nonstructural protein, NS1. The NS1 protein has the potential to damage cellular DNA through DNA nicking, covalent DNA binding, and helicase activities. We hypothesize that DNA damage induced by these activities leads to apoptosis through PARP and ATR-mediated pathways.

To investigate whether B19 is able to establish infection of hepatocytes, both primary liver cells and the hepatocellular carcinoma cell line Hep G2 were inoculated with parvovirus B19 and examined for the presence of RNA transcripts of viral genes. RT-PCR analysis demonstrated that B19 was able to infect both primary and transformed hepatocytes and produce RNA for NS1. No transcripts correlating to the structural capsid proteins VP1 or VP2 were detected. Immunofluorescence-based studies confirmed the presence of NS1 in infected cells. These experiments demonstrate that B19 enters liver cells, and that the NS1 gene is actively transcribed and the mRNA is translated in infected hepatocytes.
The ability of B19 to infect hepatocytes allows for the possibility that B19 infection of these cells is cytotoxic. Pathological studies of tissue from patients with AFLF are characterized by non-inflammatory hepatocellular death. Non-inflammatory disappearance of hepatocytes suggests apoptosis as the mechanism for B19-induced cell death in AFLF. To investigate whether B19 induces apoptosis in liver cells, hepatocytes and Hep G2 cells were infected and assayed for apoptosis by PARP cleavage assays and annexin-V staining. Infection with B19 led to PARP cleavage and induced apoptosis, with a mean of 28% of infected Hep G2 cells and 24% of infected primary hepatocytes undergoing apoptosis as determined by annexin-V staining, compared to 7% apoptosis in mock-infected cells. Several lines of evidence suggest that NS1 is the molecule responsible for the B19-induced apoptosis of liver cells. Irradiated virions, which are incapable of having their genes transcribed, do not induce apoptosis, suggesting that transcription is requisite for B19-induced apoptosis. Since NS1 is the only known transcript produced by B19 infection of hepatocytes, it is likely that NS1 is the apoptosis-inducing factor.

Apoptosis can proceed through many different mechanisms. These pathways are regulated by the involvement of different caspases, cysteine proteases that mediate apoptosis. Caspase 9 transduces apoptotic signals originating within the cell, such as responses to DNA damage. Caspase 8 transduces apoptotic signals that are initiated by ligation of TNF superfamily receptors. Caspase 3 is a target of caspases 8 and 9, and acts to carry out the final activities of apoptosis. Caspase inhibition studies demonstrated that caspases 3 and 9, but not caspase 8, are required for B19-induced apoptosis. The requirement for caspase 9 in B19-induced apoptosis suggests that apoptosis is initiated by
an internal signal, such as DNA damage. The dispensability of caspase 8 suggests that fas and TNF-α are not involved in B19-induced apoptosis. The DNA modifying activities of NS1 could allow NS1 to induce apoptosis by damaging cellular DNA.

To directly examine the apoptosis-inducing effects of NS1, the NS1 gene was cloned and fused to the gene for eGFP in an inducible vector. This vector was transfected into Hep G2 cells. Expression of the NS1 fusion protein was sufficient to cause apoptosis in a caspase-3 and–9-dependent manner, similar to the apoptosis induced by infection with whole virions. Immunoprecipitation experiments revealed that NS1 covalently binds to cellular DNA, a process which likely leads to activation of DNA damage response pathways.

Bulky adducts induce apoptosis through a pathway requiring a DNA damage repair molecule, ATR. The single-stranded DNA binding protein RPA initiates the ATR-dependent pathway by binding complexes containing ATR, leading to ATR phosphorylation. Immunofluorescence staining demonstrated that the DNA damage-sensing molecule RPA colocalizes with NS1 in transfected cells. Inhibition of ATR with caffeine decreases apoptosis in NS1-transfected cells by 70%, decreasing the percentage of apoptotic transfected cells from 37% to 12%, and implicating the DNA adduct repair pathway in NS1 induced apoptosis.

The ATR-mediated pathway for DNA damage-induced apoptosis is not the only potential DNA repair pathway induced by NS1. NS1 nicks viral genomes, causing single-strand breaks. The cellular pathway that repairs single-strand breaks can also lead to apoptosis. This pathway is initiated by the single-strand nick binding protein PARP. Upon activation, PARP poly(ADPribose)ylates neighboring proteins. Although the
mechanisms of PARP-mediated DNA repair and apoptosis are not well understood, PARP activation can lead to apoptosis through changes in mitochondrial membrane permeability, leading to activation of caspase 9. To investigate whether NS1 activates PARP, NS1 was immunoprecipitated from transfected cells and found to be poly(ADPribose)ylated. Poly(ADPribose)ylation of NS1 confirmed that PARP is active in NS1-transfected cells, and in contact with NS1 protein. When PARP was inhibited by 5-aminoisoquinolinone, NS1-induced apoptosis decreased by 57%. PARP activation in the presence of NS1 demonstrates the presence of DNA single strand nicks in the immediate vicinity of NS1.

These experiments demonstrate the cytotoxic nature of B19 infection of hepatocytes, and elucidate the mechanisms of B19-induced apoptosis of liver cells. The evidence shows that NS1 binds to DNA in a covalent manner and that NS1 expression induces apoptosis through the single-strand nick and adduct DNA damage repair pathways.

A strong association between B19 and AFLF allows treatments for AFLF to be targeted to B19. Since B19 can be neutralized with intravenous immune globulin (IVIG), it is possible that administration of IVIG could abrogate liver injury in cases of AFLF. The knowledge that B19 induces apoptosis through NS1-induced DNA damage also suggests potential therapies. PARP inhibitors have been proven useful in combating apoptosis-related injury due to ischemia, and it is possible that they would also prevent cell death induced by B19 infection. These therapies have the potential to limit the liver damage during B19-associated AFLF, perhaps eliminating the need for transplantations.
In addition, many other diseases are associated with B19 infection, and these diseases may also benefit from anti-viral or anti-apoptotic therapies.
TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................... x

ABBREVIATIONS USED IN THE TEXT. ............................................................................. xi

CHAPTER 1: REVIEW OF THE LITERATURE .....................................................................1
   A.  Introduction ........................................................................................................... 2
   B.  Parvoviruses ...................................................................................................... 3
   C.  Transcription and replication ............................................................................. 4
       1.  Transcription ................................................................................................... 4
           a. Promoter activity ......................................................................................... 4
           b. Splicing and transcripts ............................................................................. 6
   D.  DNA Replication .................................................................................................. 7
       1.  Cellular requirements for DNA replication ................................................. 11
       2. Viral factors .................................................................................................... 13
   E.  B19 Proteins ........................................................................................................ 14
       1.  NS1 ..................................................................................................................... 14
           a. Functions of NS1 ....................................................................................... 15
           b. NS1 in viral DNA replication ..................................................................... 16
           c. NS1 and viral transcription ........................................................................ 17
           d. Regulation of NS1 .................................................................................... 19
           e. Regulation of NS1 by phosphorylation .................................................... 19
           f. Regulation of NS1 by macromolecular binding ....................................... 20
           g. NS1 Structure and specific domains ......................................................... 22
       2.  VP2 ..................................................................................................................... 24
       3.  VP1 ..................................................................................................................... 25
   F.  Parvovirus B19 binding and entry ........................................................................ 26
       1.  Globoside ......................................................................................................... 26
       2. Virus binding .................................................................................................. 28
       3. Internalization ................................................................................................. 29
   G. Tropism .................................................................................................................. 31
   H.  Disease manifestations .......................................................................................... 35
       1.  Aplastic crisis .................................................................................................. 35
       2. Erythema infectiosum ...................................................................................... 36
       3. Autoimmune disease ..................................................................................... 38
       4. Liver Disease .................................................................................................. 39
   I.  Cellular and molecular pathogenesis ................................................................... 46
       1.  Cell cycle arrest ............................................................................................... 47
       2. Apoptosis .......................................................................................................... 48
           a. Apoptosis in response to DNA damage ................................................... 52
           b. ATR-mediated response to adducts ........................................................... 52
           c. PARP-mediated response to single-strand nicks ...................................... 53
A. Parvovirus B19 causality of acute fulminant liver failure ................................. 171
B. NS1 expression and apoptosis ........................................................................ 176
C. B19 infection and DNA damage ..................................................................... 179
D. Clinical implications ...................................................................................... 184
E. Conclusion ...................................................................................................... 189

LITERATURE CITED ............................................................................................ 191
LIST OF FIGURES

Figure 1.1  Rolling hairpin model of parvovirus DNA replication ........................................... 9
Figure 1.2  Hepatic manifestations of B19 infection ................................................................. 41
Figure 1.3  Activation of ATR by bulky adduct damage and subsequent induction of apoptosis ................................................................. 54
Figure 1.4  Mechanism of single-strand nick-induced apoptosis ............................................. 59
Figure 2.1  Synchronization of Hep G2 cells .............................................................................. 77
Figure 2.2  RT-PCR analysis of infected hepatocytes ............................................................... 80
Figure 2.3  Immunofluorescence of NS1 protein ................................................................. 82
Figure 2.4  B19 infection of Hep G2 Cells induces apoptosis as demonstrated by PARP cleavage ................................................................. 85
Figure 2.5  B19-induced apoptosis is viral dose-dependent ................................................... 87
Figure 2.6  B19 infection induces apoptosis in Hep G2 cells .................................................... 89
Figure 2.7  B19 infection induces apoptosis in primary hepatocytes ......................................... 94
Figure 2.8  Time course of B19–induced apoptosis of Hep G2 cells ......................................... 97
Figure 2.9  Hepatocyte growth factor abrogates B19-induced apoptosis in primary hepatocytes ......................................................................... 99
Figure 2.10  Caspase 3 is activated by B19 infection .............................................................. 103
Figure 2.11  Apoptosis induced by B19 in Hep G2 cells is dependent on caspase 3 and 9 activity, but not caspase 8 ................................................. 107
Figure 2.12  Inhibition of caspases 3 and 9 abrogates apoptosis in primary hepatocytes ................................................................. 109
Figure 3.1  Plasmid map of eGFP/NS1 ........................................................................ 123
Figure 3.2  Amino acid sequence of cloned NS1 ...................................................................... 126
Figure 3.3  True-color fluorescence image of transfected cells ............................................. 128
Figure 3.4  Western Blot and immunoprecipitation analysis of expressed NS1 fusion protein ......................................................................... 130
Figure 3.5  Cellular localization of eGFP/NS1 ................................................................. 132
Figure 3.6  Colocalization of eGFP/NS1 with nuclear factor PCNA ......................................... 135
Figure 3.7  Expression of eGFP/NS1 induces apoptosis in Hep G2 cells .................................. 137
Figure 3.8  Caspases 3 and 9 are required for NS1-induced apoptosis ........................................ 142
Figure 3.9  TNF-α and fas are not involved in NS1-induced apoptosis of hepatocytes ................................................................. 144
Figure 3.10  DNA is covalently bound to NS1 protein ............................................................... 148
Figure 3.11  DNA adduct repair pathways is necessary for NS1-induced apoptosis ................................................................. 153
Figure 3.12  eGFP/NS1 colocalizes with RPA ................................................................. 156
Figure 3.13  p53 is not necessary for B19-induced apoptosis .................................................... 159
Figure 3.14  PARP activity is necessary for optimal NS1-induced apoptosis .................................. 162
Figure 3.15  NS1 is Poly(ADP ribose)ylated ............................................................................ 164
Figure 4.1  Model for NS1-induced apoptosis ........................................................................ 182
Figure 4.2  Model for generation of anti-DNA antibodies in B19 infection ......... 187
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ADV</td>
<td>Aleutian disease virus</td>
</tr>
<tr>
<td>AFLF</td>
<td>acute fulminant liver failure</td>
</tr>
<tr>
<td>AFLF-AA</td>
<td>acute fulminant liver failure with associated aplastic anemia</td>
</tr>
<tr>
<td>AIF</td>
<td>apoptosis inducing factor</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>APAR</td>
<td>autonomous parvovirus replication</td>
</tr>
<tr>
<td>APAF-1</td>
<td>apoptosis promoting and initiating factor</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>AT mutated and Rad3 related</td>
</tr>
<tr>
<td>ATRIP</td>
<td>ATR interacting protein</td>
</tr>
<tr>
<td>B19</td>
<td>parvovirus B19</td>
</tr>
<tr>
<td>BFU-E</td>
<td>blast forming unit-erythroid</td>
</tr>
<tr>
<td>BH</td>
<td>Bcl-2 homology</td>
</tr>
<tr>
<td>BRDU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>CFU-E</td>
<td>colony forming unit-erythroid</td>
</tr>
<tr>
<td>CPV</td>
<td>canine parvovirus</td>
</tr>
<tr>
<td>ddTTP</td>
<td>dideoxythymine triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EI</td>
<td>erythema infectiosum</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FPV</td>
<td>feline parvovirus</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>g/dl</td>
<td>grams per deciliter</td>
</tr>
<tr>
<td>G1 phase</td>
<td>growth 1 phase</td>
</tr>
<tr>
<td>G2 phase</td>
<td>growth 2 phase</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>IAP</td>
<td>inhibitor of apoptosis</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin 6</td>
</tr>
<tr>
<td>kd</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>moi</td>
<td>multiplicity of infection</td>
</tr>
</tbody>
</table>
mRNA ................................................................. messenger RNA
MVM ................................................................. minute virus of mouse
NAD ................................................................. nicotinamide
NF-kappa B ........................................................... nuclear factor kappa B
NLS ................................................................. nuclear localization signal
nm ................................................................. nanometer
NS1 ................................................................. nonstructural protein 1
nt ................................................................. nucleotides
NTP ................................................................. nucleotide triphosphate
ORF ................................................................. open reading frame
PARP ................................................................. poly(ADP ribose)polymerase
PBS ................................................................. phosphate-buffered saline
PCNA .............................................................. proliferating cell nuclear antigen
PCR ................................................................. polymerase chain reaction
PIF ................................................................. parvovirus initiation factor
PKC ................................................................. protein kinase C
PS ................................................................. phosphatidylserine
RA ................................................................. rheumatoid arthritis
RNA ................................................................. ribonucleic acid
RPA ................................................................. replication protein A
RT-PCR ........................................................ reverse transcription polymerase chain reaction
S-phase ........................................................ synthesis phase
SAP ................................................................. shrimp alkaline phosphatase
SLE ................................................................. systemic lupus erythematosus
SDS ................................................................. sodium doedecyl sulfate
SDS-PAGE ...................................................... sodium doedecyl sulfate-polyacrylamide gel electrophoresis
TNF-α ............................................................. tumor necrosis factor alpha
TNFR1 ............................................................. tumor necrosis factor receptor 1
UV ................................................................. ultraviolet
VP1 ................................................................. viral protein 1
VP2 ................................................................. viral protein 2
µg ................................................................. microgram
µl ................................................................. microliter
µM ................................................................. micromolar
Chapter 1

Review of the Literature
A. Introduction

Although infection with Parvovirus B19 is widespread, with approximately 60% seropositivity in adults worldwide, years passed before any disease was associated with B19 infection. In 1981, B19 was recognized as the causal agent of transient aplastic crisis in patients with sickle cell disease or other hemolytic disorders\(^{14,16}\). Later, B19 was identified as the causative agent of fifth disease, a rash illness of children\(^{10}\). Since that time, B19 has been implicated in a variety of diseases, ranging from hematological disorders to hepatitis to a wide spectrum of autoimmune diseases.

One organ targeted by B19 is the liver. It is possible to recover B19 DNA from liver samples, and a variety of studies link B19 infection and liver disease. A number of investigators have associated B19 infection with liver disease. The reported clinical findings vary from mild elevation of liver enzymes to total acute fulminant liver failure (AFLF). Acute fulminant liver failure is a life-threatening event that often requires liver transplantation. Although there are multiple reports demonstrating B19 DNA in diseased livers, it is also possible in some instances to find B19 DNA in healthy liver. Also, B19 is unable to replicate in liver cells\(^{8}\), raising the question of how B19 infection might cause liver disease.

To understand the possible mechanisms for B19-induced liver damage, it is important to understand the transcription strategy, replication cycle, and cell tropism of B19. Cellular factors influencing transcription and DNA replication of B19 influence the susceptibility of the cell to productive B19 infection. It is also imperative to understand the function of the viral nonstructural protein, NS1, which is likely the
molecule responsible for the cytopathic effects of B19. NS1 has multiple DNA modifying activities, and could lead to apoptosis through damaging cellular DNA.

**B. Paroviruses**

B19 is a member of the family parvoviridae, which consists of viruses with a linear, single-stranded DNA genome. Paroviruses are the only known viruses that contain a linear-single stranded DNA genome and infect animals. Paroviruses are extremely small (11-22 nm) and do not possess an envelope. The host range of each individual parovirus is usually limited; however, members of the family parvoviridae infect a range of animals from mammals to insects. Some important paroviruses that infect mammals are adeno-associated virus (AAV), canine parvovirus (CPV), feline parovirus (FPV), minute virus of mouse (MVM) and Aleutian disease virus (ADV), which infects mink. AAV is classified in the genus dependovirus, and requires the presence of a helper virus, such as adenovirus or a herpesvirus, in order to replicate, while the other paroviruses are capable of autonomous replication in the proper cell type.

A major determinant of permissiveness for parovirus infection is the ability of the cell to undergo cell division. Unlike many other DNA viruses, all of the paroviruses are extremely dependent on the host DNA replication machinery and will only establish productive infections in dividing cells. Paroviruses lack mechanisms to induce the host cell to initiate S phase, and so need the host cell to divide of its own accord in order to replicate viral DNA. B19 has a still more limited tropism with productive infection occurring only in erythroid precursors.
C. Transcription and Replication

1. Transcription

a. Promoter activity

Transcriptional regulation at least partially controls which cells are permissive for B19 infection. The genome of B19 contains multiple open reading frames (ORF)s, including four of significant size\textsuperscript{168}. All transcripts are initiated from a single strong promoter, named the P6 promoter because it is found at map unit 6 of the B19 genome. The use of a single promoter is in contrast to all other known autonomous parvoviruses, which utilize two promoters, one for the structural and one for the nonstructural proteins, and the dependoviruses, which use three promoters. The P6 promoter was discovered using \textit{in vitro} transcription assays to test putative promoter elements. The minimal elements of the promoter that this group found are a TATATATA sequence and a start site 31 nucleotides downstream. The P6 promoter is very strong, being at least fivefold stronger than the SV40 promoter\textsuperscript{15,52}. There are Sp1 transcription factor recognition sites (GGCGGG) 26 and 38 nucleotides upstream of the TATA sequence\textsuperscript{15}. Gel-shift assays demonstrated that at least one cellular factor, most likely Sp1, binds to these sites. Mutation of the GGCGGG region leads to a profound loss of transcription from the p6 promoter\textsuperscript{14}.

In addition to the Sp1 binding sites, the terminal hairpin contains regulatory sequences that enhance promoter activity. Truncation of the hairpin sequence revealed an enhancer element between nucleotides -249 to -157. The site was examined by DNase
footprinting using HeLa cell extracts and cloned B19 DNA, which revealed a repeated sequence of CCGGAAGTCCGCC. Competition studies showed that Sp1 does not bind to the sequence in the hairpin, implicating other cellular factors in the regulation of B19 transcription\(^9\). Gel shift analysis revealed that YY1, Sp1, Sp3, ets related factors, oct-1 and oct-2 all bind to specific sequences in the B19 promoter\(^{10,15}\), and in vitro transcription assays demonstrated enhancement in the presence of YY1. The relative amounts of each of these factors may regulate the P6 promoter activity. Sp1 and Sp3 are known to compete for binding sites and are regulated according to the cell cycle\(^{70,86,122}\).

In addition, cell extracts from the erythroleukemia cell line K562 formed unidentified complexes with the promoter region that were not found in the nonpermissive cell lines HeLa or BJAB\(^{151}\). These findings implicate specific enhancement of the P6 promoter in permissive cells, especially since the P6 promoter is 25 times more active in K562 cells than in HeLa Cells\(^{67}\).

Regulation of the P6 promoter is important for viral infectivity. By restricting transcription to certain points of the cell cycle, regulation allows maximal expression of NS1 at times when it is most useful to the virus and least likely to kill the cell prematurely. Cell-cycle dependent regulation may also prohibit accumulation of capsid proteins before the DNA is available to become packaged into the capsids. The favoring of NS1 over capsid proteins in nonpermissive cells could serve several purposes, or it may be the reason the cells are nonpermissive. Since NS1 causes cells to die by apoptosis, as will be demonstrated in chapter 3, expression of NS1, but not the capsid proteins, in cells unsuited for viral replication may diminish the antigenic load and mask infection from the immune system until productive replication occurs.
b. Splicing and transcripts

B19 infection results in at least 9 polyadenylated transcripts. B19 utilizes only 1 active promoter, so RNA splicing generates the multiple mRNA species required for B19 function. Of the 8 polyadenylated transcripts found in B19 infected cells, 5 correspond to the nonstructural protein NS1 and the 2 capsid proteins VP1 and VP2, as demonstrated by hybridization with specific probes\textsuperscript{134}. Primer extension analysis demonstrates that all transcripts start with a small 50 base pair leader sequence corresponding to the nucleotides directly after the P6 promoter\textsuperscript{134}. The presence of this small sequence again confirms that all B19 transcription is initiated at the p6 promoter, in contrast with other parvoviruses. The mRNA transcripts coding for VP1 and VP2 contain the leader sequence spliced to a sequence mid-genome, which in turn is spliced to the RNA containing the coding region for the protein. Each structural protein has 2 transcripts which contain the entire sequence; the difference in length is caused by utilization of multiple splice donor sites in the mid-genome sequence.

Four of the 9 identified transcripts do not code for the major proteins. The 2 smallest transcripts are coded from the extreme 5’ end of the genome. Proteins with molecular masses of 11 kilodaltons have been identified which correspond to translation products from these mRNAs. However, these proteins are dispensable for viral replication and their function is not known\textsuperscript{183}. Two transcripts, which share a polyadenylation signal with NS1, do not contain an initiation codon and no protein has been identified to correspond with them.
The transcript for NS1 is terminated by an unusual polyadenylation signal, ATTAAA or AATAAC. This polyadenylation sequence, which is used in only 1-11% of eukaryotic genes, is associated with tissue specificity\textsuperscript{134,184}. Alternative splicing is a major mechanism through which NS1 regulates gene expression. The splicing patterns are cell specific, implying the utilization of specific cellular factors. Splicing may be the major reason for the cell tropism of B19, but exactly how splicing of B19 transcripts is regulated remains to be determined.

D. DNA Replication

Parvoviruses are unique in being the only animal virus with a single stranded linear DNA genome. To prime their genomic DNA for replication, parvoviruses use extensive hairpins consisting of imperfect palindromes on each end of the genome. These hairpins fold back upon themselves, providing a self-priming origin of replication. Parvoviruses must use the cellular DNA replication machinery to replicate their DNA. Unlike many other DNA viruses, however, parvoviruses do not contain a mechanism to force host cells into S phase, where DNA replication occurs. Productive replication of parvoviruses is therefore limited to cells that are already undergoing division.

The ends of parvovirus genomes consist of an extensive region of self-complementary sequence which folds back on itself to form a T-shape. The inverted repeats solve a fundamental problem in replicating linear DNA. Since all DNA polymerases need a free 3' OH group to initiate DNA synthesis, a primer is necessary, usually made of RNA. Since there are no DNA polymerases that proceed from the 5' end, the end of the DNA strand hybridized to the primer would be lost, leading to
repeated shortening of the DNA strand. The inverted repeats solve this problem by providing a self-priming region where DNA synthesis can originate. By utilizing a "rolling hairpin" approach, multiple full-length copies of the genome can be produced in the form of a concatamer.

Peter Tattersal proposed the rolling hairpin DNA replication strategy in 1976\textsuperscript{193}. The rolling hairpin model suggests that DNA replication is initiated at the 3' end of the genome, which is double-stranded due to the palindromic inverted repeat. The 3' end of the genome thus serves as a primer, with strand elongation continuing to the 5' end of the DNA. Due to the palindromic nature of the 5' end of the B19 genome, upon being copied the daughter strand self-anneals to form a hairpin, allowing the use of the recently generated strand as a template for further replication, which proceeds by strand displacement to the original 3' end of the genome (Figure 1.1). The result is a concatamer consisting of one template strand and one complementary strand of DNA, covalently linked at one end. Through the further use of strand displacement, this basic replicative unit could continue to grow until it contained a large concatamer of alternating genome and complementary strands\textsuperscript{193}. The rolling hairpin model is attractive because it explains the ability of parvoviruses to maintain their genomic integrity, and explains the necessity for the nicking activity of NS1 in parvoviral genomic replication. The nicking of DNA by NS1, along with being essential for parvoviral replication, may be a major method of pathogenesis due to its DNA damaging potential.
Figure 1.1. Rolling hairpin model of parvovirus DNA replication.  

A. Input DNA exists in a mostly single stranded form with terminal hairpins.  

B. DNA replication initiates at the 3’ end of the DNA strand and proceeds to the opposing hairpin. *De novo* synthesized DNA is shown as dashed lines. 

C. DNA polymerase displaces the hairpin and makes a complimentary copy.  

D. Terminal hairpins refold  

E. DNA synthesis continues, displacing the original DNA strand and using the newly synthesized complementary DNA strand as a template.  

F. DNA synthesis displaces and copies the right side hairpin, then uses the original DNA strand as a template to generate another complementary copy. Refolding of hairpins allows continuation of the replication process.
Figure 1.1
1. Cellular requirements for DNA replication

Paroviruses are extremely dependent on host cell replication components to replicate their genomes. An essential property of all cells that support paroviral replication is the ability to initiate the cell cycle. The conversion of the input single stranded DNA to a double stranded replicative form is S-phase dependent, as was shown by quantifying viral DNA in highly synchronized cells infected with minute virus of mouse. Viral DNA replication does not occur until early S-phase, regardless of when infection occurs. In bovine parvovirus infection, cellular proteins were found that complexed with bovine parvovirus DNA; however, these proteins were not present in the cell until S phase, suggesting one mechanism through which the cell cycle can regulate paroviral DNA replication. Studies using nuclear lysate systems defined the cellular proteins that are important for paroviral replication. DNA polymerase α was demonstrated to be important in both bovine parvovirus and H-1 parvovirus replication, and polymerase γ is also required. Kinetic studies using the specific DNA polymerase inhibitors aphidicolin and ddTTP suggested that the initial conversion of single stranded to double stranded DNA, including strand displacement synthesis is carried out by polymerase α, and then viral DNA replication proceeds using polymerase γ.

Further data implicates polymerase δ in paroviral genome replication. The DNA replication process is dependent on proliferating cell nuclear antigen (PCNA), as addition of exogenous PCNA allowed for MVM DNA replication in cells with the cell cycle blocked by p21. The ability of PCNA to rescue paroviral DNA replication suggests that DNA polymerase δ is important in the replication process. Polymerase δ requires PCNA.
for processivity. The implication that polymerase \( \delta \) is involved in viral DNA replication is strengthened by the findings that the viral NS1 protein colocalizes with PCNA, polymerase \( \delta \), RPA-1, and RFC, all components of the polymerase delta complex. In MVM infected cells, polymerase \( \delta \) is not activated until S-phase of the cell cycle, providing another explanation for why viral DNA production is limited to S phase\(^{10,47}\). Polymerase \( \delta \) is involved in DNA repair, and the involvement of the polymerase \( \delta \) complex suggests that the B19 genome is recognized as damaged DNA by the cell. Alternately, the finding that NS1 colocalizes with proteins involved in DNA repair may indicate a DNA damaging activity of NS1.

In addition to polymerases, other cellular factors are required for replication of parvoviral genomes. Single stranded DNA binding protein and PCNA are required for \textit{in vitro} replication\(^{32}\). A factor, termed parvovirus initiation factor (PIF) is also necessary for MVM replication. This factor binds to ACGT motifs and cooperates with the viral NS1 protein in initiating DNA synthesis\(^{33}\). An additional factor was discovered to be necessary for NS1 nicking of the origin in HeLa cell extracts. The protein was putatively identified as a member of the high mobility group 1/2 family. PCNA is involved in DNA repair and synthesis. Recruitment of these molecules to the site of DNA replication suggests that DNA repair mechanisms are required for parvoviral DNA replication. Two pathways that control DNA repair are the ATR and PARP mediated responses, which are involved in the adduct and single-strand nick repair pathways, respectively. These pathways can also initiate apoptosis, and may be involved in the cytopathic effect of parvovirus infection.
2. Viral factors

The 2 elements necessary for DNA replication contributed by the B19 virus are the palindromic terminal repeats and the NS1 protein. NS1 coordinates the replication and transcription of the virus, and the palindromic terminal repeats provide a mechanism for priming the viral DNA for replication.

In MVM DNA replication, sequences exist within the palindrome that are essential for full DNA replication capacity. Therefore, the physical hairpin is not the only important factor in the makeup of the terminal repeat. There are three mismatched nucleotides within the repeat in both MVM and B19, forming a small bubble within the hairpin. MVM genomes without this bubble are replicated at a much lower rate than genomes containing the bubble. The sequence of the mismatches does not seem to be important, however, since restoration of the mismatches with altered sequence restores full replication competency\(^41\). Further research demonstrated that the bubble in the 3' hairpin of MVM acts as a site for the viral NS1 protein to nick the DNA, and that it is at that point that DNA replication is initiated. NS1 also becomes covalently attached to this site after nicking\(^44\). The inverted repeats in B19 are different than those of MVM in that they are identical on both the 3' and the 5' end, while in MVM the ends are distinct. In the structure of the terminal hairpins, B19 is more similar to AAV than MVM, although the AAV repeats are only 145 nucleotides long. The repeats of the B19 genome are 383 nucleotides in length. The palindrome is not perfect, with four nucleotides at the hinge region and three nucleotides on each strand unpaired\(^50\). The identical nature of the repeats in B19 suggests that nicking by NS1 and initiation of replication can occur at either end, as opposed to MVM, which requires both terminal palindromes for effective
replication but only initiates replication at one site\textsuperscript{191}. The presence of DNA motifs that are thought to bind as yet unidentified cellular proteins are also important in parvoviral replication\textsuperscript{23,40}. It is unknown whether NS1 is capable of nicking and binding to cellular DNA as well as viral DNA.

E. B19 Proteins

The structure of B19 is simple. The virion consists of two coat proteins in icosahedral formation with a triangulation number of one, meaning that the configuration of the capsid is as simple as an icosahedral virus can be. The capsid surrounds the single stranded DNA genome. The two major structural proteins, VP1 and VP2, make up the viral capsid while NS1 is a nonstructural protein that coordinates many of the replicative functions of the virus. A family of small, alternatively spliced 11 kd proteins of unknown function are also present in B19 infected cells\textsuperscript{183}.

Immunoprecipitation of B19 capsids from iodinated viremic serum with antibodies from seropositive donors demonstrates that VP2 accounts for approximately 90\% of the viral protein\textsuperscript{42}. In MVM, an autonomous parvovirus similar to B19, NS1 becomes covalently attached to the viral DNA\textsuperscript{31,45}, and so may be present in extremely low amounts in infectious virions.

1. NS1

NS1 is a 71 kilodalton, slightly basic protein\textsuperscript{42}. Although it is named the nonstructural protein, there is actually a copy which is attached to the viral DNA and localizes to the outside of the virion\textsuperscript{45}. Relatively little is known about the structure and
function of the nonstructural protein of B19. Given the lack of an infectious clone of B19, it is extremely difficult to perform molecular studies on the functions of NS1. The cytotoxic nature of NS1 also contributes to the difficulty in studying it, since transfected cells quickly die. Much of the knowledge of the functions of NS1 comes from analogy with the nonstructural proteins of the minute virus of mouse, MVM, and adeno-associated virus, AAV. It must be noted, however, that B19 differs significantly from the nonstructural proteins of other parvoviruses. The most obvious difference is that MVM and AAV have two nonstructural proteins, while B19 has only one, which seems to perform the tasks of both nonstructural proteins found in MVM and most other parvoviruses. Additionally, the B19 and MVM nonstructural proteins are only twenty-six percent identical, with thirty-six percent similarity. Given the similar functions of the nonstructural proteins among parvoviruses, however, knowledge may be gained through analogy. A transfection vector utilizing cloned NS1 under the control of an inducible promoter would allow for greatly enhanced understanding of the functions of NS1. One such vector will be described in chapter 3.

a. Functions of NS1

NS1 performs a diverse array of functions. Purified NS1 protein from Aleutian disease virus (ADV), an autonomous mink parvovirus, binds to and cleaves ATP, and acts as a helicase in an ATP-dependent manner. The ATPase activity is stimulated in the presence of single-stranded DNA. Proteins with helicase activity unwind DNA, leading to the generation of single stranded areas in the DNA. The helicase activity of NS1 aids in the replication of the viral genome. NS1 is also required for replication of the
parvovirus genome, is involved in creating the viral origin of replication, and is a potent transcription factor for both viral and cellular genes.

b. NS1 in viral DNA replication

NS1 is absolutely required for parvoviral DNA replication. MVM variants with temperature sensitive mutations in NS1, as well as dominant negative mutations of NS1 in AAV have been generated. In the presence of either of these mutations, no viral DNA synthesis occurs\(^{26,195}\). The requirement for NS1 in DNA replication is not due to its activity as a transcription factor, since certain mutant NS1 forms that block genomic DNA replication are nevertheless able to activate transcription of the viral genes. Instead, NS1 is essential for the process of initiating DNA synthesis. Paroviral genomes exist in the cell in the form of double-stranded replicative intermediates, consisting of the input genomic DNA complexed to a complementary, \textit{de novo} synthesized copy. In order for genomic replication to take place, the DNA must undergo a conformational change, with the palindromes folding back on themselves instead of being complexed to their complements in the replicative form. NS1 stimulates this change by nicking and binding to the genomic DNA at the origin of replication. The helicase activity of NS1 partially unwinds the replicative form, and DNA synthesis proceeds. The newly synthesized DNA strand folds over on itself, generating the hairpins that are essential for parvoviral DNA replication\(^{202}\). This model for NS1-regulated DNA replication is supported by findings that the helicase activity of NS1 controls viral replication\(^{124}\). Along with initiating genomic replication, NS1 is responsible for separating individual copies after
DNA replication. NS1 nicks the multi-genome concatameric replication intermediate, releasing individual B19 genomes.

NS1 interacts with the DNA replication machinery throughout the many forms the B19 DNA takes and the many stages of B19 genome replication. NS1 enters the cell covalently linked to the DNA, nicks the double stranded replicative form to allow replication to begin, modifies the topography of the DNA with using helicase activity and separates the replicated viral genomes. Without NS1, parvoviral DNA replication could not occur. The DNA-modifying activities of NS1 suggest the possibility that NS1 may also modify cellular DNA. If NS1 were to nick and unwind cellular DNA, it would create areas that could be recognized as DNA damage by the single stranded nick binding protein, PARP, and the bulky adduct repair pathway, regulated by ATR.

c. NS1 and viral transcription

In addition to regulating B19 genome replication, NS1 is an enhancing transcription factor for the B19 promoter. In the presence of NS1, viral gene transcription increases substantially. Infection of the semipermissive cell line UT-7, a megakaryoblastoid leukemia-derived line, demonstrated early expression of NS1 transcripts. Northern blot analysis of infected cell extracts showed that NS1 mRNA is produced 6 hours after infection, while the mRNA for VP1, VP2 and the small RNA transcripts did not appear until 20 hours post infection. The early production of NS1 allows it to regulate the transcription of the genes for the structural proteins. The addition of NS1 protein to in vitro transcription assays using the P6 promoter enhanced transcription of a reporter gene. Cotransfection of a vector containing NS1 and a P6-
driven reporter plasmid confirmed that the enhancement due to NS1 occurs \textit{in vivo} as well as \textit{in vitro}\textsuperscript{53}. Deletion mutations involving the DNA surrounding the P6 promoter demonstrated that NS1-responsive elements exist between nt 100 and 160, and 233 and 298. With these regions deleted, NS1-induced enhancement of transcription was lost\textsuperscript{67}.

Interactions of cellular transcription factors with NS1 are also important in gene activation. NS1 forms a complex with the transcription factors SP1 and SP3, as demonstrated by gel shift analysis. NS1 and SP1/SP3 bind directly to B19 DNA at regions near the promoter\textsuperscript{152}, and their interactions may alter the conformation of the DNA, or act to recruit other molecules to the promoter. The presence or absence of these cellular factors may determine whether a cell is permissive or not for B19 infection.

The transcription-enhancing effects of NS1 are not specific to B19 genes alone. Cotransfection of NS1-expressing plasmids with HIV-LTR-responsive luciferase reporter plasmids led to a striking increase in activity from the LTR, with at least a fourfold increase over tat, the HIV-transactivating protein, alone\textsuperscript{180}. NS1 is also capable of enhancing transcription of cellular genes. Stably transfected cell lines with inducible NS1 production show activation of the IL-6 transcription upon induction of NS1. The effect is dependent on a NF-κB binding site in the IL-6 gene, suggesting as yet undiscovered interactions between NS1 and cellular proteins\textsuperscript{105}. NF-κB is a pleiotropic transcription factor, affecting such processes as apoptosis and cellular activation. Interactions between NS1 and NF-κB have, therefore, the potential to dramatically influence the activity or life of the cell.
d. Regulation of NS1

Regulation of the many functions of NS1 is crucial for a successful viral infection. Considering the cytotoxicity of parvoviral nonstructural proteins, indiscriminate activation may kill the cell before the virus has time to replicate its DNA, synthesize the capsid proteins, and exit the cell. Most of the current understanding of the regulation of NS1 is by analogy with the NS1 protein from MVM, and these findings may or may not correlate to B19. In MVM, NS1 is a phosphoprotein that is regulated by at least two processes: phosphorylation and binding to other macromolecules. NS1 can homodimerize, bind to cellular proteins, and bind to DNA, all of which affect its activity.

e. Regulation of NS1 by phosphorylation

Nuesch and colleagues performed experiments to see whether the phosphorylation state of MVM NS1 has any effect on its activities. They purified vaccinia-virus expressed NS1, and dephosphorylated serine and threonine residues with calf intestinal alkaline phosphatase. They then used this hypophosphorylated protein in in vitro assays examining the ability of NS1 to bind to and nick viral DNA, helicase activity, and ATPase activity. They found that binding to the recognition element on the viral DNA was not impaired by hypophosphorylation; instead hypophosphorylated NS1 was better able to bind to DNA. The ability of NS1 to nick DNA, however, was found to be five to tenfold less in the hypophosphorylated than the normal protein. Helicase activity was severely decreased in the hypophosphorylated NS1, with a 30-fold drop in efficiency. The ability of NS1 to hydrolyze ATP was also markedly lower, with an eight-fold
decrease in efficiency. Protein Kinase C was able to rephosphorylate the hypophosphorylated NS1, and restore full helicase activity\textsuperscript{125}.

The majority of the phosphorylation of NS1 occurs in the helicase domain\textsuperscript{37}, suggesting that phosphorylation may play a direct role in DNA unwinding. The \textit{in vitro} experiments were later confirmed using an \textit{in vivo} system. A dominant negative mutant of PKC $\lambda$ was coexpressed with NS1, preventing phosphorylation. Hypophosphorylated NS1 was unable to initiate viral DNA replicative events\textsuperscript{129}.

The next task of NS1 phosphorylation researchers was to identify the exact kinases that are important for regulation of NS1 in MVM. Multiple groups found that protein kinase C was essential for restoring the ability to direct viral genome replication in an \textit{in vitro} system. The specific isoforms were later identified as PKC $\lambda$, and PKC $\eta$\textsuperscript{51,124,128}. PKC $\eta$ was found to aggregate in the nucleus in response to MVM infection, suggesting that NS1 may not only be a target of PKC phosphorylation, it may regulate its activity\textsuperscript{90}. This finding bears out a study showing that NS1 is capable of interfering with the phosphorylation of cellular proteins\textsuperscript{7}.

The kinases that regulate NS1 are in turn regulated by such factors as the cell cycle. Phosphorylation control of NS1 may control such events as nuclear localization and the ability of NS1 to induce apoptosis.

\textbf{f. Regulation of NS1 by macromolecular binding}

For optimal activity, NS1 binds to a variety of molecules. NS1 in MVM forms an oligomer in the cytoplasm\textsuperscript{130}. Using a yeast two-hybrid system, Pujol and colleagues generated a peptide that specifically interferes with NS1 oligomerization. Addition of the
interfering peptide to \textit{in vitro} helicase reactions or \textit{in vivo} infected cells led to a suppression of NS1 activity\textsuperscript{150}. The inactivation of NS1 upon inhibition of the ability to homooligomerize demonstrates that self-assembly is necessary for NS1 activity, as is the case for many other helicases\textsuperscript{150}. In the case of B19, it is unknown whether NS1 forms homodimers, a question which will be discussed in chapter 3.

In addition to homodimerization, NS1 also binds to cellular factors. The NS1 proteins from both MVM and H-1 parvovirus (a rodent virus) form specific bodies in the nucleus, called APAR bodies, for Autonomous Parvovirus Replication Bodies. These bodies contain the cellular replication elements PCNA, small glutamine rich TPR-containing protein, and survival motor neuron proteins. Interestingly, cyclin A is found in APAR bodies, suggesting an interaction between cyclins and NS1. BRDU incorporation colocalizes with APAR bodies, demonstrating that APAR bodies are sites of active DNA synthesis\textsuperscript{47,212}. These bodies probably serve as sites of genome replication in infected cells. An interaction between cyclins and NS1 may help to explain the effects of NS1 on the cell cycle. It is not clear how the elements of the APAR bodies are recruited. They may physically interact with NS1, or they may be drawn to the sites of DNA damage induced by the nicking or helicase activities of NS1.

One of the principal functions of NS1 is to separate the viral genomes from the concatameric replication intermediate. For NS1 to function as a nicking enzyme, however, it needs to bind to cellular components. A transcription factor, named parvovirus initiation factor (PIF) binds to ACGT sites on the paroviral genomic DNA. PIF also interacts with NS1, strengthening the interaction of NS1 with the genomic DNA. In the absence of PIF, NS1 is unable to nick the DNA, suggesting the necessity for at
least a trimolecular complex in order for nicking to occur. The necessity for specific complex formation is important when it is considered that NS1 serves not only as a viral nickase and helicase, but also as a transcription factor and therefore binds DNA in multiple sites. Indiscriminate nicking of the single stranded genome could lead to irreparable cleavage of the viral DNA.

**g. NS1 structure and specific domains**

Parvoviral nonstructural proteins exhibit a wide range of activities and effort has been made to identify the regions of the polypeptide responsible for each of these activities. A major role of NS1 is to separate viral genomes from concatameric replication forms. Often, DNA nicking enzymes utilize coordinated metal atoms for activity. A putative metal coordination site was located in AAV, suggesting a possible site of enzymatic activity. Mutation of this site rendered the vaccinia virus-expressed NS1 protein unable to nick DNA. The metal, if there is one, coordinated by parvoviral nonstructural proteins is not known.

Analysis of the peptide sequence of NS1 reveals a probable purine nucleotide-binding site in the most conserved region of the protein. A lysine to histidine mutation at this site in AAV generates a temperature-sensitive mutant. AAV virions with the temperature-sensitive mutation exhibit dominant negative behavior, suppressing their own replication and that of superinfecting wild type AAV at the nonpermissive temperature. Cells infected with mutated virus demonstrate an increase in the amount of NS1 found in the cell, indicating that cellular levels of NS1 are autoregulated. Mutations of the same region in MVM induce a phenotype with an inability to
transactivate viral genes or nick the replicative forms of viral DNA\textsuperscript{80,126}, confirming the importance of this site to the function of NS1.

Mutation of the homologous site in B19 leads to a striking decrease in toxicity of NS1, as measured by the ability of transfected cells to form colonies\textsuperscript{109}. These results demonstrate that the nucleotide-binding motif is essential for practically every function of the nonstructural proteins.

An additional motif in parvoviral nonstructural proteins is their nuclear localization signal. NS1 is primarily a nuclear protein. In MVM, NS1 localizes to the nucleus two hours after transcription\textsuperscript{43}. The nuclear localization signal in MVM is made up of a four amino acid sequence, KKKI. Mutation of this sequence completely abolishes the ability of NS1 to translocate to the nucleus. However, cotransfection of mutant and wild type NS1 causes both mutant and wild type NS1 to localize in the nucleus, suggesting that NS1 is transported to the nucleus in a complex consisting of at least two NS1 molecules. Interestingly, mutations in the ATP binding region, such as discussed above, also eliminated the ability of NS1 with a functional NLS to transport other NS1 molecules with nonfunctional nuclear localization signals. This suggests that dimerization is energy dependent or requires a conformational change brought on by ATP binding\textsuperscript{130}. Although the evidence is good that NS1 from MVM has only one nuclear localization signal, similar mutation of the homologous region of NS1 protein from B19 failed to abrogate nuclear localization, suggesting that in B19, NS1 may have a different or multiple nuclear localization signals. Most of the functions of NS1 are nuclear, suggesting that nuclear localization would be essential for its activity.
NS1 is a multifunctional protein with a variety of DNA modifying effects. Many of the effects of NS1 are potentially dangerous to the cell. The ability of NS1 to damage cellular DNA has not been well established, but will be discussed in chapter 3 of this work.

2. VP2

VP2 and the minor capsid protein, VP1, make up the capsid of B19. The two proteins are identical except that VP-1 contains an N-terminal extension of 226 amino acids. VP2 is a slightly basic protein with a molecular weight of 56 kilodaltons. When expressed in a baculovirus or Chinese hamster ovary expression systems VP2 will self-assemble to form empty capsids, without the need for VP1 or viral DNA. Coexpression of VP1 with VP2 in these systems yields a more natural empty capsid. This capsid is similar to naturally occurring virions when examined by immunoassay and the ability to stimulate a neutralizing antibody response. However, the empty capsids are difficult to crystallize, and even when crystallization is achieved, X-ray diffraction analysis does not yield high resolution diffraction data. The inability to form good crystals may be caused by instability induced by the lack of DNA in the structure.

X-ray diffraction patterns have been achieved at 8 angstroms resolution, and it is possible to glean some information from these about the structure of VP2. The dominant structural feature of VP2 is an antiparallel eight stranded beta sheet, a common domain found in isosahedral viruses that infect vertebrates. Other features of probable importance are a canyon at the threefold axis and the unusual morphology of the fivefold axis, where VP1 is located. Most parvoviruses have a hole at the fivefold axis, through
which the DNA is thought to extrude. B19 lacks this pore, implying that the process of DNA uncoating may be different in B19 than other parvoviruses. The principal difference between B19 and other parvoviruses is in the threefold axis of symmetry, where the antigen-binding pocket is located in canine parvovirus. In canine parvovirus, there is a large spike at the threefold axis, which is entirely absent in the B19 structure.

Deletion studies using empty capsid generation systems are a valuable tool in understanding the function of VP2 epitopes. The amino acids 25-39 are essential for capsid formation, as deletions in this area abrogate the ability of the VP2 to self-associate or form capsids. VP2 seems to fulfill its main purpose by containing the viral DNA. However, many of the properties and functions of VP2 are still undefined, such as the exact location of the antigen-binding site and interactions with the genome.

3. VP1

VP1 has a molecular weight of 83 kilodaltons. In the virion, VP1 is likely found at the fivefold axis of symmetry. Although in canine parvovirus, a related member of the parvovirus family, the protein region corresponding to the VP1 terminus is buried inside the capsid, the unique region in B19 is exposed on the surface of the virion. The external location of VP1 was demonstrated by specific neutralizing antibody studies, and also studies using recombinant empty capsids containing truncated VP1. Despite containing the entire sequence of VP2, VP1 will not self-aggregate, although a truncated form without the N-terminal unique region will form empty capsids, and coexpression of VP1 and VP2 will form capsids containing both proteins. Another difference between VP1 found in parvovirus B19 and other parvoviruses is that the fivefold axis of
canine and feline parvovirus forms a pore structure, while the fivefold axis of B19 is closed.

VP1 is found in and contributes to the structure of the virion, and also has enzymatic activity. The region spanning amino acids 130 to 195 contains a phospholipase A2 motif. Expression of cloned VP1 fragments yielded an active phospholipase A2 enzyme. The carboxy terminus of the VP1 unique region was sufficient for enzymatic activity; however, when the entire unique region was assayed, the enzymatic activity was much greater. Mutational analysis of MVM, which contains homologous phospholipase A2 domains, indicates that phospholipase activity is not necessary for attachment or viral entry, but is important in transfer from the late endosome to the nucleus. The exact mechanisms through which phospholipase A2 activity facilitates nuclear transfer are not known. It is possible that lipid messengers are generated by the phospholipase A2 activity, or that this activity cleaves glycolipids that bind to B19.

F. Parovovirus B19 Binding and Entry

1. Globoside

The cellular receptor for B19 is globoside, or blood group P antigen. The identification of globoside as the receptor initiated with the observation that B19 will hemaglutinate red blood cells. This discovery had been long in coming since the major source of B19 capsids is viremic serum, and IgM in the serum neutralizes the
hemaglutinating activity. It was only by using repeatedly frozen serum and recombinant empty capsids that the ability of B19 to aggregate red cells was found\textsuperscript{20}.

Many parvoviruses agglutinate red blood cells by binding to glycolipids, so it was hypothesized that the B19 receptor was also a glycolipid. Lipid preparations from red blood cells inhibited hemaglutination by B19, and a variety of enzymatic digestions were carried out to determine the identity of the inhibiting factor. The molecule that was found to inhibit B19 hemaglutination was globoside, a neutral glycosphingolipid, with six sugar residues attached to a lipid, which is inserted into the plasma membrane. Globoside is abundant on red blood cells and red blood cell precursors\textsuperscript{19}.

Studies of individuals with a complete lack of globoside further confirmed that globoside is the receptor for B19. Serum from these people indicated that none had antibodies to B19 infection, although the prevalence of anti-B19 antibodies in control populations is high. Also, bone marrow colonies from globoside-deficient individuals were unaffected by B19, while bone marrow cells from control donors were infected and inhibited in colony formation by incubation with B19\textsuperscript{21}. Since globoside is abundant on red blood cells but had not at that time been identified on other tissue types, it was thought that globoside was the determining factor in cell tropism. Globoside was later shown to be present on a variety of cell types\textsuperscript{36}, eliminating receptor availability as the determining factor in B19 tropism. Liver cells, for example, express globoside, but are nonpermissive for B19 infection.
2. Virus Binding

The first step in the viral infection cycle is binding to the cell. Studies by Saikawa et al.\textsuperscript{160} demonstrate that antisera raised to empty capsids containing only VP2 will not neutralize B19 infectivity. However, antibodies against a peptide consisting of the unique region of VP1 will both block virus infection and agglutinate wild type virus particles\textsuperscript{160}. These data suggest that the principal epitopes of B19 important for infectivity are located in the unique region of VP1. However, another study using a panel of antibodies showed a failure of many (8 out of 9) neutralizing monoclonal antibodies to recognize linear epitopes of either VP1 or VP2, instead binding to conformationally dependent epitopes\textsuperscript{209}. The antibody that did recognize a linear epitope bound to a region of VP2. Still further studies have demonstrated that the junction between the VP1 unique region and the VP2-VP1 common region is a target for neutralizing antibodies\textsuperscript{160}.

In most parvoviruses, the epitope responsible for receptor binding lies in a canyon, or deep pocket in the surface of the virus, at the threefold axis of the virion, a location occupied by VP2 in B19. Cryoelectron microscopy studies of B19 empty capsids and empty capsids bound to globoside show the ability of the canyon at the threefold axis to bind the receptor, probably at a density of three globoside molecules per unit. Furthermore, monoclonal antibodies that neutralize B19 infectivity and hemaglutination are located around the probable receptor-binding site\textsuperscript{29}. These data suggest that the area at the threefold axis of the B19 capsid is responsible for receptor binding.

There are several possibilities to explain why the unique region contains neutralizing epitopes when it does not seem to bind globoside. A coreceptor, β integrin,
has recently been identified for B19 binding\textsuperscript{201}, and the unique region of VP1 may be involved in coreceptor binding. Since the unique region extends beyond the normally flat surface of the virion, it may be involved in preliminary attachment in order to bring the virion close enough to the cell to bind the low-lying globoside. VP1 influences the conformation of the entire capsid. Conformation-specific anti-B19 antibodies will bind empty capsids containing VP1, but not empty capsids formed from VP2 only\textsuperscript{159}. The conformational change brought about by the inclusion of VP1 may lead to the uncovering of neutralizing epitopes. Another explanation for the neutralizing nature of anti-VP1 antibodies is that the antibody interferes with the phospholipase A2 activity of VP1, which has been reported to be essential for infectivity\textsuperscript{54}.

3. Internalization

After binding to globoside on the cell surface, the virion must enter the cell and proceed to the nucleus in order for gene transcription and DNA replication to occur. Although there is very little known about B19 specifically, inferences can be drawn from the mechanisms used by other autonomous parvoviruses such as canine and feline parvoviruses. CPV entry was examined by electron microscopy. Virions were taken into the cell in small vesicles, which frequently fused. Many viruses, such as influenza, require vesicle acidification in order to escape into the cytoplasm. Treatment of infected cells with chloroquine or ammonium chloride, both of which prevent acidification of the lysosome, prevents CPV from entering the cytoplasm, instead leading to the development of large vesicles containing multiple virions\textsuperscript{8}. The inability of CPV to enter cells in the absence of lysosomal acidification demonstrates a lysosomal pathway for viral entry.
Further studies revealed that treatment with nocodazole, which depolymerizes microtubules, also blocks CPV entry, suggesting a role for microtubule-mediated transfer of virus-containing vesicles. These researchers also directly microinjected CPV into the cytoplasm in order to determine if virus-altering events occurred in the vesicles. Both natural and acid (pH 5.0) treated virions were unable to establish infections when microinjected, suggesting that an event other than mere acidification, such as proteolysis of some viral components, occurs in the vesicles.

In order to further define the entry of CPV particles into cells, electron microscopy and temperature sensitive dynin mutant cell lines were used to demonstrate that CPV was taken up through clathrin-coated pits in a receptor, rather than a fluid, specific manner, and was dependent on dynein for internalization. These researchers also discovered that the block in infectivity seen with chloroquine and ammonium chloride is probably less dependent on lysosomal pH and more on the correct transport of the lysosome to the late lysosomal compartment. This was discovered using bafilomycin A1, an agent that has a minor effect on lysosome acidification but prevents merging with the late lysosome.

Taken together, these data indicate a specific, receptor-mediated uptake of parvovirus particles, followed by the need for specific trafficking of the virion to the late lysosome, where modifications to the capsid occur, leading to infectivity of the viral DNA. B19 probably follows a similar pattern. The ability of pharmacologic agents to inhibit uptake suggests targets for the treatment of serious effects of B19 infection.
G. Tropism

Although B19 is implicated in AFLF, liver cells do not support replication of B19. B19 will only replicate in erythroid precursors, and only at a specific stage of erythroid progression. The reasons for the strict restriction of B19 replication to these cells, although once thought to be simply a matter of receptor expression, is actually complex, involving at a minimum the processes of viral entry, transcription, splicing, polyadenylation, DNA replication and translation.

The finding that B19 was responsible for cases of transient aplastic crisis in patients with hemolytic anemia led to the hypothesis that B19 was replicating in red blood cell precursors. Attempts to propagate B19 in bone marrow cultures proved successful if the erythroid cell stimulating factor erythropoietin was added to the cultures. B19 infection of bone marrow cultures selectively reduced the number of erythroblasts and normoblasts from 70% to 10% of cultured cells, while in control cultures the number of erythroid precursors only decreased to 50% of cells. B19 DNA replication and the production of intact virions were detected in this bone marrow system.

Subsequent studies using infection of bone marrow cultures to identify the exact cells infected and the pathology of B19 infection revealed that the destruction of erythroid cells begins 2 days post infection and continued until the erythroid cells were depleted. Infected erythroblasts displayed altered morphology, with large size, immature chromatin, and no hemoglobination. The presence of nuclear inclusion bodies was also prevalent in infected cells.

Analysis of blood and bone marrow from infected volunteers confirms the in vitro findings, with the presence of decreased hemoglobin (2-3 g/dl decrease) beginning at day
2 post-infection, and almost total absence of erythroid precursors in the bone marrow 10 days post-infection\textsuperscript{147}. Further in vivo studies using Southern blotting analysis demonstrated the presence of B19 replicative forms in the granulocytic fraction of circulating cells of infected patients. This finding extended the tropism of B19 from the marrow to cells that are found both in the bone marrow and in circulation\textsuperscript{89}.

Hematopoiesis in fetuses occurs in the liver, where the erythroid precursors reside before becoming found predominantly in the bone marrow. Cultures using fetal liver-derived erythroid precursors allow B19 replication at levels up to 100 times those found in cultures derived from adult bone marrow\textsuperscript{22}.

In an effort to identify the exact cell(s) in which B19 replication occurs, Srivastava and Lu enriched bone marrow for erythroid precursors by depleting out adherent cells and T cells and using Ficoll gradients to isolate low-density cells\textsuperscript{182}. These cells were then sorted using flow cytometry to isolate My10++, DR+ cells. These erythroid precursors were infected with B19, along with unfractionated and partially fractionated bone marrow extracts serving as controls. The investigators then examined the fractions for DNA and the ability to form colonies of either granulocytic or erythroid cells. Granulocytic progenitors were unaffected, but the CFU-E and BFU-E cells were equally susceptible to B19 infection\textsuperscript{182}. These results demonstrate the extreme specificity of B19 for erythroid lineage cells. The cells along the erythroid pathway demonstrate increasing susceptibility to B19 infection with increasing differentiation\textsuperscript{188}, with normoblasts being extremely susceptible\textsuperscript{163,167}. These results identify partially differentiated red blood cell precursors as the only normally occurring adult cells in which B19 has been found to replicate.
There have been numerous attempts to establish stable cell lines permissive for B19 replication. The absence of success in this area is startling, since erythroid-derived cell lines that mimic the host cell of B19 are available, and many paroviruses show a pronounced tropism for transformed cells due to their rapidly dividing nature. Although no attempt at generating cell lines for productive B19 infections has met with complete success, the failures and partial successes still provide information about what is required for productive B19 infection.

Attempts to create cells in which B19 will replicate involve treating erythroleukemia or megakaryocytic leukemia-derived cell lines with erythropoeitin to induce differentiation of the erythroid pathway, and then infecting the cells. Although B19 replication has been demonstrated in numerous cell lines, including MB02 and UT-7/Epo, the yield of viral particles from these cells has been very low, with the output approximately equal in titer to the inoculating dose. In contrast, in bone marrow culture systems 1000-fold increases in output virus have been reported. The term semipermissive has been applied to cell lines in which replicative forms have been detected, to differentiate from nonpermissive cells in which there is no evidence for viral DNA replication.

The establishment of semipermissive cell lines helps to address the question of why B19 is so specific for its particular cell type. The factors thought to be most important for paroviral cell tropism, receptor availability and division of the target cell, are both available in these cell types, and yet the low levels of virus produced point to other cellular factors as being important in the life cycle of B19. As discussed in the section dealing with B19 transcription, B19 transcription is more effective when extracts
from semipermissive cells are used than when nonpermissive cell extracts are used in \textit{in vitro} transcription assays. Cell-specific, differentially regulated transcription factors such as AP-1/AP-3 and YY1 have been demonstrated to bind to B19 DNA and influence transcription. The polyadenylation of the B19 NS1 transcripts may also play a part, as the polyadenylation signal used by B19 transcripts is only functional in certain cells. Disruption of polyadenylation could have drastic effects on B19 replication, whether it leads to either excessive or insufficient translation of NS1 mRNA. Excessive NS1 could kill the cells before DNA replication or capsid assembly could be completed, leading to a lack of productive infection. Insufficient NS1 protein would also not allow replication, as NS1 is required for efficient transcription from the p6 promoter, initiation of DNA replication, and resolution of the viral genome from its concatameric intermediate form. NS1 may also have other, unknown functions that could influence replication.

Differential splicing of B19 transcripts has been suggested as an explanation for the limited tropism of B19. Brunstein and co-workers found that in the semipermissive cell line MBO2 a majority of the transcripts were aberrantly spliced, utilizing a nontraditional splice donor site\textsuperscript{24}. The altered splicing pattern extended the length of the leader sequence that initiates all B19 transcripts, causing the inclusion of the translational start site for the NS1 protein. The splice donor follows immediately after the splice start site, linking it with a sequence containing multiple in-frame stop codons, potentially terminating translation\textsuperscript{24}. Translation usually does not initiate at downstream AUG codons, so this splicing pattern would prevent the structural proteins from being formed, explaining at least in part the lack of virions produced in nonpermissive infection.
Understanding the factors involved in cell tropism is important when evaluating the role of B19 in AFLF. Although B19 cannot replicate in the adult liver, B19 infection has been clearly linked to multiple types of hepatitis. A possible explanation for this discrepancy is that B19 may be able to infect liver cells, but not complete its replication cycle. The knowledge that NS1 is predominantly expressed in nonpermissive cells suggests that NS1 may be involved in the effects of B19 infection of liver cells, and other non-erythroid cells.

**H. Disease Manifestations**

Although B19 productively infects only erythroid precursors, only one of the many clinical manifestations associated with B19 is directly caused by destruction of bone marrow cells. Other clinical symptoms serve to illustrate the sequelae of the immune response to B19 infection and the fact that much of the pathogenic potential of B19 is not well understood.

**1. Aplastic crisis**

Aplastic anemia is a disease where the production of red blood cells is insufficient, often necessitating transfusion. The lack of red blood cells is caused by a deficiency in red blood cell precursors in the bone marrow. Aplastic crisis was the first disease associated with B19. The first report linking B19 to aplastic crisis concerned several cases that presented at a hospital in Britain. Viruses with parvoviral morphology were isolated from serum samples from these patients. The connection between B19 and aplastic anemia was confirmed in Jamaica among patients with sickle-cell anemia.
Twenty-eight patients with aplastic crisis were identified in 1979. 86% of these patients seroconverted to B19 positivity at the time of or shortly after their aplastic crisis. In patients with sickle cell anemia but no aplastic crisis, only 4% were positive for B19 antibodies. The correlation between B19 and aplastic anemia seems to be that patients with sickle cell anemia are unable to produce sufficient red cells when B19 infection stops progression of the erythroid precursors.

2. Erythema infectiosum

Erythema infectiosum (EI), also known as fifth disease, is a generally mild disease that most often occurs in children. EI is a two-phase disease, with the first symptoms being fever, myalgia, headache, itching and general malaise. These symptoms resolve and are followed two weeks later by an erythematous maculopapular rash. The rash generally begins on the face, and then spreads to the trunk and limbs. It commonly fades in the center, leading to a raised outer edge and lacy appearance. The rash often is concurrent with the development of arthralgia, or joint pain, which may be acute or chronic in nature. Stimuli such as exposure to sunlight or bathing will often bring on recurrence of the rash.

B19 was first proposed to be the causative agent of EI in 1983. A group studying an outbreak among schoolchildren in London found evidence of B19-specific IgM antibody in serum samples from 31 out of 31 cases of EI. Although this group did not include a control population in this report, their previous work demonstrated that specific anti-B19 IgM is rarely found in asymptomatic control populations. Other reports subsequently strengthened the association of EI with B19. Researchers examining an
outbreak of EI in Japan found that 34 out of 34 EI patients were seropositive for anti B19 IgG and IgM. Only 15% of the 141 control patients had anti-B19 IgG, with none of the controls being seropositive for anti-B19 IgM. This group also found that the levels of anti-B19 IgG were higher in EI patients than in seropositive controls131. Still another group found that 24 out of 26 patients diagnosed with EI had anti-B19 IgM, while none of 33 controls had specific anti-B19 IgM antibodies207.

To understand how contagious B19 is, these investigators screened family members of EI patients. They found that 61% of households with a patient with anti-B19 IgM also had at least one other member with IgM anti-B19 antibodies. This suggests that B19 is transmissible by casual contact207.

In addition to serological evidence, the presence of B19 virions in EI was also investigated. In one study, DNA was found by hybridization studies in the serum of a small number of EI patients, but none of the controls30. In another study, B19 DNA was detected by PCR in 98% of one 120 patients with EI, with titers decreasing rapidly one week after onset. B19 DNA was not detected in any control patients58.

To prove B19 causality of EI, nine volunteers were inoculated intranasally with dilutions of B19. Four of these volunteers were seropositive for B19 IgG before inoculation. B19 DNA was detected by hybridization in 4 of the 5 seronegative volunteers, with the only non DNA-positive seronegative volunteer being the one to receive the lowest dosage of virus. DNA levels peaked 8 days post-inoculation and were undetectable by hybridization in all patients by day 17. All 4 seronegative volunteers who developed viremia also developed specific IgM antibodies to B19 between days 8
and 13 post-inoculation. Three of the 4 initially seronegative volunteers with viremia developed EI, with a rash beginning on day 17 and lasting 2 to 3 days\textsuperscript{5}.

These studies demonstrated conclusively that B19 was the causal agent for EI. Due to the biphasic nature of the illness, it was hypothesized that the rash and arthralgia aspects of B19 infection are mediated by the immune response to B19, rather than by direct cytopathic effect of B19 infection, especially since B19 DNA is in decline by the time rash symptoms occur\textsuperscript{58}. The hypothesis that these sequelae of B19 infection are immune-mediated is supported by the findings of inflammatory T-cell infiltrates in the skin tissue comprising the rash\textsuperscript{118}, although B19 virions are also detectable\textsuperscript{164}. The exact pathogenesis of the rash illness is not understood, but probably involves both T and B cell mediated factors.

3. Autoimmune disease

B19 infection has been implicated in a variety of autoimmune diseases. Adults infected with B19 often develop arthropathy that mimics the symptoms of rheumatoid arthritis (RA) and may last for months to years. B19 infection is linked to rheumatoid arthritis beyond shared symptoms. Historical evidence demonstrates that rheumatoid arthritis and B19 appeared in Europe at about the same historical period. Further, molecular studies demonstrate that B19 infection can cause invasiveness in synoviocytes, a phenotype common in RA, and that B19 infection leads to increases in IL-6 and TNF-\(\alpha\), both important cytokines in the pathogenesis of RA.

B19 is also potentially involved in another autoimmune disease, systemic lupus erythematosus (SLE)\textsuperscript{78}. B19 infection is frequently succeeded by the production of
autoantibodies, including anti-DNA antibodies that are a hallmark of SLE\textsuperscript{103,181}. Interestingly, patients who develop anti-DNA antibodies during B19 infection are less likely to have anti-B19 capsid antibody responses\textsuperscript{78}. This finding may explain the lack of serological data connecting lupus with B19, in that when B19 infection produces antibodies that lead to SLE, anti-capsid antibodies are not present, so demonstrating strong associations between SLE and B19 infection is difficult\textsuperscript{13}.

The role of B19 in autoimmunity is not proven. However, the numerous reports associating B19 infection with various autoimmune disorders is suggestive that B19 may influence the immune system towards autoimmunity. The interactions of the NS1 protein of B19 with DNA suggests a mechanism through which B19 infection may lead to broken tolerance to nuclear antigens, a model which will be discussed in chapter 4.

4. Liver Disease

Hiroyuki Tsuda made the first report of possible B19 involvement in liver disease, in 1993\textsuperscript{194}. He found that in two cases patients presenting with increased hepatic enzymes and fatigue were also positive for IgM against parvovirus B19. Serum from these patients tested negative for hepatitis A, B, and C viruses, cytomegalovirus, and Epstein-Barr virus,\textsuperscript{194} leading to the conclusion that B19 may have been responsible for the elevation in liver enzymes. No hepatomegaly was seen in this case, which will be shown to be consistent with the absence of liver inflammation in B19-associated hepatitis.

Since the report by Tsuda, multiple investigators have found B19 as a possible cause of hepatitis presenting in a diverse array of situations (Figure 1.2). Pinho et al., and
Yoto et al., describe cases similar to that reported by Tsuda, with increased liver enzymes, especially serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and with IgM positivity for B19 and PCR positivity for B19 DNA in the liver\textsuperscript{145,210}. B19 was associated with post-transplantation liver dysfunction\textsuperscript{92}, fibrosing cholestatic hepatitis\textsuperscript{169}, and hepatic insufficiency in newborns\textsuperscript{102}.

Of particular interest of the liver disorders associated with B19 infection is acute fulminant liver failure (AFLF). AFLF is a potentially fatal illness involving rapid failure of the liver. This condition often requires transplantation. In up to one third of cases, AFLF is associated with aplastic anemia. B19 is a predominant cause of aplastic crisis, suggesting that there may be an association between B19 and AFLF. When liver tissue from patients with AFLF associated with aplastic anemia (AFLF-AA) is examined histologically, a massive disappearance of hepatocytes is evident, without inflammatory infiltrates or fibrosis. The finding of non-inflammatory hepatocyte death suggest apoptosis as the cause of cell death.

Multiple studies show an increased prevalence of B19 DNA in livers of patients with AFLF-AA. Langnas et al found B19 DNA in 4 of 6 livers from patients with AFLF-AA, while only 5 of 34 patients with liver failure of known origin had B19 DNA in their livers. Serological studies were also suggestive in this study, with 100% of those with AFLF-AA testing positive for B19 IgG, while only 24% of the control group had positive
Figure 1.2. Hepatic manifestations of B19 infection. Findings of clinical research articles relating to B19 infection and hepatitis are presented, including the number of patients studied, the means of verifying B19 infection and the findings of the studies.
<table>
<thead>
<tr>
<th>Disease</th>
<th>Patients and Findings</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute fulminant liver failure</td>
<td>4/6 B19 DNA+ patients 5/34 B19 DNA+ controls</td>
<td>Langnas et al.91</td>
</tr>
<tr>
<td></td>
<td>4/21 B19 DNA+ patients 0 B19 DNA+ controls</td>
<td>Sokal et al.178</td>
</tr>
<tr>
<td></td>
<td>2/2 B19 DNA+ patients</td>
<td>Pardi et al.139</td>
</tr>
<tr>
<td></td>
<td>5/6 B19 DNA+ patients 5/34 B19 DNA+ controls</td>
<td>Karetnyi et al.84</td>
</tr>
<tr>
<td></td>
<td>1/1 B19 DNA+ patient</td>
<td>Alliot et al.3</td>
</tr>
<tr>
<td></td>
<td>2/2 B19 DNA+ patients</td>
<td>Granot et al.69</td>
</tr>
<tr>
<td></td>
<td>2/8 B19 DNA+ patients</td>
<td>Tung196</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>2/2 B19 IgM+ patients</td>
<td>Tsuda194</td>
</tr>
<tr>
<td></td>
<td>1/1 B19 IgM+ patient, central nervous system symptoms, B19 DNA in cerebrospinal fluid</td>
<td>Yoto et al.211</td>
</tr>
<tr>
<td></td>
<td>3/129 IgM+, B19 DNA+ nondifferentiated hepatitis patients</td>
<td>Pinho et al.145</td>
</tr>
<tr>
<td>Transplant-associated hepatitis</td>
<td>1 B19 DNA+ patient with aplastic anemia</td>
<td>Shan et al.109</td>
</tr>
<tr>
<td></td>
<td>14/49 B19 DNA+ patients also had hepatitis, 6/63 patients without B19 DNA also had hepatitis</td>
<td>Lee et al.92</td>
</tr>
<tr>
<td>Newborn hepatitis</td>
<td>1 B19 DNA+ patient with hydrops fetalis and advanced liver disease</td>
<td>Metzman et al.102</td>
</tr>
<tr>
<td>No liver disease</td>
<td>17/35 B19 DNA+ liver transplant patients 4/17 B19 DNA+ control autopsy patients</td>
<td>Eis-Hübinger et al.57</td>
</tr>
<tr>
<td></td>
<td>4/15 B19 DNA+ AFLF patients 2/30 B19 DNA+ nonviral hepatitis patients 10/30 B19 DNA+ HBV or HCV patients</td>
<td>Wong et al.205</td>
</tr>
</tbody>
</table>

Figure 1.2
anti-B19 antibody titers\textsuperscript{91}. The same group confirmed these results with another cohort of patients. In the second study, 5 of 6 patients (83\%) with AFLF-AA tested positive for B19 DNA in their liver tissue, while only 5 of 34 (15\%) patients with AFLF of known origin tested positive for B19. This study used an immune-capture-PCR technique that demonstrated the presence of both B19 DNA and capsid protein, suggesting the presence of whole virions in the liver\textsuperscript{84}. Serological and PCR studies by this group also found increased prevalence of anti-B19 IgG antibodies and DNA in AFLF patients as compared to controls, noting also multiple mutations in the B19 DNA isolated from AFLF patients\textsuperscript{117}. It is possible that these mutations allow persistence or confer upon B19 the ability to infect the liver. However, no studies have been done to evaluate these possibilities.

Other research groups have also found a correlation between B19 and AFLF. Tung et al. found that two of eight patients with AFLF-AA tested positive for B19, with the other six testing positive for known hepatitis viruses\textsuperscript{196}. They also found the prevalence of AFLF-AA to be greatly increased among children and young adults. This epidemiological pattern is suggestive of a virus that is generally acquired in childhood, such as B19.

In a study of children, Sokal et al. found B19 genomic DNA in serum samples from 4 of 21 patients with unexplained AFLF, while not detecting B19 DNA in any of their control group of patients with AFLF of known origin or biliary atresia. The liver enzyme studies were consistent with the effects previously seen in B19-associated liver disease, with high ALT, high AST, low or no increases in bilirubin and a lack of jaundice. These researchers found a significant decrease in bilirubin in B19 positive
patients compared to patients with liver disease from other causes. The patients with B19-associated hepatic failure were distinct in the course of their disease as well, with a milder course leading to spontaneous resolution within 3 weeks, and not necessitating transplant.

Other reports associating B19 with AFLF-AA include Pardi et al, who presented two cases of AFLF-AA. B19 IgM antibodies were found in both patients, along with classic signs of B19 infection such as a diffuse maculopapular rash. Granot et al also discussed two cases of AFLF-AA in which B19 DNA was detected in serum samples.

Despite the large number of studies associating B19 with liver disease, proving causality has remained difficult. Two studies have concluded that B19 does not cause disease in the liver. One, by Eis-Hubinger, investigated the prevalence of B19 in liver tissues of a randomly selected cohort of adult liver transplant patients. All of these patients were between 22 and 72 years old. Eis-Hubinger et al. also collected liver and bone marrow specimens from 23 autopsy patients selected at random. They found that out of the 43 samples taken from liver transplant patients, 17 were positive for B19 DNA in the liver, or about 40 percent. Of the randomly selected autopsy patients, 4 of 23 patients tested positive for B19 DNA in the liver, a proportion of 17 percent. The authors concluded from this study that the prevalence of persistent B19 DNA is very high, and that DNA can be found in the livers of patients without disease. They therefore questioned the studies mentioned above correlating B19 DNA in the liver with AFLF.

While the finding that B19 DNA persists for years in the liver is important, the results from this group do not contradict the results of the earlier studies. There was no attempt in the study by Eis-Hubinger to compare the status of AFLF patients with non-
AFLF patients and, in fact, only one patient with AFLF was included in the study. However, even this study, which purports to discredit the idea that B19 is involved in AFLF, found a higher proportion of B19 DNA in patients requiring liver transplants than controls. The patient age was also different than the studies performed by Langnas and Karetnyi. Most patients with AFLF-AA are children, while the group studied by Eis-Hübinger was completely made up of adults. The results are suggestive, therefore, that a primary or recent infection, such as would be found in children, has a greater potential to damage the liver than a persistent infection where B19 may have been residing in the liver for years.

Another study, by Wong and associates, also disputed the finding that B19 caused AFLF-AA. In their study, two groups of liver samples totaling 116 samples were tested for B19 DNA using PCR. These investigators found B19 DNA in 18% of all livers from the first group with an increased prevalence of DNA in livers from patients with AFLF. Twenty-four percent of all livers from the second group were positive for B19 DNA, with an increased prevalence of B19 DNA in patients with AFLF as compared to other known causes of liver disease, such as biliary atresia. Interestingly, the prevalence of B19 in hepatitis associated with hepatitis B or C was similar to that of AFLF. The higher prevalence of B19 DNA in AFLF did not reach statistical significance in the first group and was similar to the prevalence of B19 in known hepatitis infections; therefore the authors claim that there is no relationship between B19 DNA and AFLF.

Ironically, considering the conclusions of Wong et al, there was in their own data a significant increase in the prevalence of B19 DNA when the categories of liver disease were separated. Thirty-five percent of liver samples from AFLF tested positive for B19,
while only 5% of livers with biliary atresia tested positive for B19\textsuperscript{205}. Excluding the known cases of hepatitis B or C, these data actually strengthen the case for B19 causality of AFLF. The reason for the increase of B19 DNA prevalence in patients with hepatitis B or C is not known. However, other viruses have been shown to enhance activity of the B19 promoter\textsuperscript{146}, and it is possible that hepatitis B or C viruses enhance B19 infection.

B19 is capable of remaining in tissues for years. It is, therefore, possible to find B19 DNA in the liver without disease being present. The ability to persist, which is very common among DNA viruses, complicates Koch's postulates, the standard rules for determining causality. B19 further complicates these rules by being unable to grow in pure culture and having a tropism specific for humans, invalidating the idea of causing disease in an animal. Difficulties with the standard methods of proving causality in the case of B19 limit the standard that can be applied to the question of whether or not B19 causes liver disease to a "preponderance of the evidence." Under this standard, if a large amount of data linking B19 to liver disease is generated, B19 should be considered the causal agent until it can be definitively proven to be so or a better explanation can be developed. There is an ever-increasing body of evidence that reveals a role for B19 in hepatic disease. In order to understand the role of B19 in liver tissues, it is necessary to examine the cellular interactions of B19 with liver cells. Finding that B19 is able to infect and damage liver cells would provide significant support for the idea that B19 causes liver damage in natural infection.

I. Cellular and Molecular Pathogenesis

Bone marrow specimens from B19-infected patients are marked by the presence
of B19-infected, apoptotic giant pronormoblasts, a specific type of erythroid precursor. The fact that the cells are arrested at the pronormoblast stage indicates a possible block to the cell cycle.

DNA damage is possibly the mechanism that initiates cell cycle arrest and apoptosis, since the nicking and helicase activities of NS1 could damage DNA. Cells respond to DNA damage in different ways depending on the type and extent of the damage and the type of repair that is indicated. Nicks, for example, are recognized by PARP, which ribosylates neighboring proteins and initiates the process of DNA repair. However, if the nicks are extensive, PARP can initiate apoptosis by causing the translocation of apoptosis inducing factor from the mitochondria to the nucleus. Double strand DNA breaks and bulky adducts, in contrast, are resolved by the kinases ATM and ATR, which initiate a kinase cascade leading to a blockade in the cell cycle until the damage is fixed. If the damage is too extensive to be repaired, apoptosis can ensue. NS1 generates single-strand breaks through nicking activities and if bound to chromosomal DNA would be present in the form of bulky adducts. These activities of NS1 could activate the PARP and ATR pathways, respectively.

1. Cell cycle arrest

Two studies by Eiji Morita and colleagues examined the effect of B19 infection on the cell cycle. They found that infected cells undergo arrest at the G2 phase, without progressing to M phase\textsuperscript{111}. By irradiating infectious virions, they were able to differentiate between the effects of transcription products, principally NS1, and material contained in the virion, such as genomic DNA or capsid proteins. Transfection of the
NS1 gene allowed the study of the effects of NS1 directly. Although infection with B19 induces G2 arrest, B19 infection also induces G1 arrest when a mitotic inhibitor, paclitaxel, is added. The arrest at G1 is dependent on transcription of viral genes, since irradiated B19 does not induce arrest at G1 phase. Interestingly, the irradiated input virus still causes cell cycle arrest at G2 phase, indicating that a component of the virion, and not NS1 production, is responsible for this arrest. Transfection with NS1 produced cell cycle arrest at G1 phase, and p21 was upregulated in NS1-transfected cells\textsuperscript{110}. These findings indicate that both virion components and de novo translated NS1 are capable of interfering with the cell cycle.

Other parvoviruses also cause cell cycle arrest. Cells transfected with NS1 from MVM arrest at G1, S, and G2 phases. The regulatory protein p53 is necessary for arrest at S and G2 phase. p21, however, which is also involved in regulating the cell cycle, is only necessary for the arrest at G2 phase\textsuperscript{49}. ADV infection of feline kidney cells blocks the cell cycle in cells expressing NS1, but cells that do not express NS1 continue through the cell cycle\textsuperscript{133}. The mechanisms of the NS1-induced cell cycle arrest are unknown. The potential DNA-damaging effects of NS1 may induce DNA lesions, the response to which could interrupt the cell cycle.

2. Apoptosis

Apoptosis is the name for programmed cell death, in contrast to necrosis, which is uncontrolled cell death. Apoptosis is an active form of cell death requiring energy, and is marked by several unique processes, including loss of membrane polarity, nuclear
fragmentation, DNA condensation, and DNA cleavage. Apoptotic cells are normally quickly phagocytosed by macrophages without inflammation.

Apoptosis can be initiated by a variety of stimuli. Such events as growth factor withdrawal, DNA damage, hypoxia, and cytokine stimulation can all signal the cell to undergo apoptosis. Apoptosis is mediated through the actions of caspases, proteases that cleave after an aspartate residue. Specific caspases are activated in response to different apoptotic signals. There are three main caspase-mediated pathways for apoptosis. The first occurs as a result of stimulation through the TNF-Receptor 1 (TNFR1) superfamily, which includes such molecules as fas and TNF-receptor 1. Binding of the appropriate ligands to these receptors causes trimerization, bringing the death domains on the receptors in close contact with each other. The death domains recruit other proteins, such as caspase 8, containing death effector domains. Upon association, caspase 8 cleaves itself, producing the active form\(^{114}\). Activated caspase 8 cleaves caspase 3\(^{185}\), an effector caspase which is responsible for many of the actual processes of cell death.

The second apoptotic pathway is transduced through the mitochondria. The mitochondrial pathway is activated by cellular stress, including growth factor withdrawal and DNA damage. Members of the Bcl-2 family of proteins control apoptosis mediated through the mitochondria\(^{38}\). The Bcl-2 protein family is characterized by the presence of Bcl-2 homology region (BH) domains. BH domains are found in both anti-apoptotic molecules and pro-apoptotic molecules. The anti-apoptotic molecules contain BH 1-4 regions, and include Bcl-2, Bcl-X\(_L\), Bcl-w, and Mcl-1. The pro-apoptotic members of the Bcl-2 family are divided into two groups. One group, the Bax sub-family, contains BH regions 1-4 like the antiapoptotic molecules. This subfamily includes the molecules Bax,
Bak and Bok\textsuperscript{38}. The second group of proapoptotic proteins, the BH3-only subfamily, consists of proteins that contain only a BH3 group. There are numerous members of this protein family, and different molecules from this family are involved in initiating apoptosis as a result of specific stimuli.

The BH3-only proteins are most likely involved in transducing specific apoptotic signals. For example, p53 or p73 enhance transcription of the BH3-only subfamily members Puma and Noxa in response to DNA damage, while the BH3-only subfamily members Bad and Bim become activated in response to cytokine withdrawal\textsuperscript{38}. BH3-only proteins bind to the prosurvival members of the Bcl-2 family, primarily through the BH3 domain, although membrane targeting domains may be involved in optimal binding\textsuperscript{38}.

Binding of BH3 only proteins to BCL-2 antiapoptotic proteins allows activation of the Bax-like proteins and apoptosis\textsuperscript{38,165}. An understanding of the exact mechanism through which the antiapoptotic family members control activation of the Bax-like proteins has been elusive, although there are several models that seek to explain the relationships involved. One model proposes that Bcl-2 antiapoptotic molecules sequester BH3-only molecules, maintaining them in an inactive state. Increased concentrations of active BH3-only molecules, then, occupy more binding sites on antiapoptotic Bcl-2 proteins. Once all of the binding spaces are occupied, the BH3-only molecules then bind to and activate Bax-like molecules. A problem with this model is that the BH3-molecules have not been shown to bind to Bax or Bak. Another model suggests that the molecule Bid, which is sequestered by BCL-2, is released upon binding of BH3-only family members by BCL-2. Bid then binds to and activates Bax-like proteins. However,
Bid has not been demonstrated to bind to Bax or Bak, and Bid deficient mice seem normal. This model may still be correct, however, if the role of Bid is redundant and another protein can activate Bax-like proteins in the absence of Bid. A third model proposes that Bcl-2 controls activation of Bax-like proteins through alternate mechanisms, such as calcium flux or caspase activation\(^3\).

Activation of the Bax-like protein family leads to mitochondrial membrane permeability changes. To initiate these changes, Bax and Bak form homo-oligomers, which disrupt the outer cell membrane, allowing the release of cytochrome C and other potentiators of apoptosis such as Smac/Diablo, Omni/HtrA2, and apoptosis inducing factor (AIF)\(^6\). Although the exact mechanism of membrane disruption is controversial, it is probable that Bax-like protein oligomers form pores in the outer membrane\(^6,7\).

Cytochrome C interacts with a protein called APAF-1, leading to the formation of a large, multiprotein complex known as the apoptosome. APAF-1 contains a CARD domain, which, upon binding cytochrome C, becomes available to bind to and activate caspase 9\(^9\). Caspase 9 activates caspase 3, which carries out the end stages of apoptosis\(^7\). Inhibitors of apoptosis, or IAPs, bind to pro-caspases and prevent their activation. Smac/Diablo and Omni/HtrA2, which are released from the mitochondria along with cytochrome C upon Bax-like protein oligomerization, bind to and inhibit IAPs, allowing apoptosis to proceed\(^1\).

The third apoptotic pathway is not well characterized, but is involved in apoptosis due to stresses such as excessive misfolding of proteins. This pathway is transduced through changes in the endoplasmic reticulum. The principal caspase involved in the ER-
induced apoptosis pathway is caspase 12. Upon activation, caspase 12 activates
caspase 9, leading to apoptosis.

Caspase 3 is a target of caspases 8, 9, and 12, and so all three apoptotic pathways
come together at the activation of caspase 3. Caspase 3 cleaves caspases 6 and 7, which
complete the process of apoptosis. Caspase 3 also cleaves PARP, leading to an inhibition
of DNA repair.

a. Apoptosis in response to DNA damage

DNA can sustain multiple types of damage, and there are specific repair
mechanisms to deal with the different types of damage. NS1 could induce two types of
DNA damage, single strand nicks and bulky adducts. Single strand nicks would be
generated with NS1 nicking and helicase activity, and bulky adducts would be generated
if NS1 covalently links itself to DNA. Both nicks and adducts can lead to apoptosis if the
damage is extensive.

b. ATR-Mediated Response to Adducts

Bulky adducts are repaired by the short-patch excision repair pathway. This
pathway is initiated by the nuclear factor RPA binding to the damaged area. RPA has a
greatly increased affinity for damaged or single-stranded DNA over undamaged or
double-stranded DNA. Fluorescence anisotropy studies revealed a 15-fold increase in the
affinity of RPA for DNA with a single-stranded region over normal DNA. RPA also
has a higher affinity for damaged double-stranded DNA than undamaged double-stranded
DNA, binding to cisplatin-induced DNA adduct damage. The increased affinity for
cisplatin-damaged DNA occurs because RPA partially unwinds the damaged area, exposing single-stranded DNA for RPA to bind. Through binding to damaged DNA, RPA acts as a DNA damage sensor. Binding of RPA to the damaged DNA allows recruitment of multiple proteins, including ATRIP. ATRIP recruits and activates ATR.

ATR is a major mediator of DNA damage repair. When activated, ATR phosphorylates a variety of substrates, including Chk-1 and Chk-2. These molecules phosphorylate the cell-cycle control protein p53. Chk-1 and Chk-2 also phosphorylate the apoptosis-inducing proteins p63 and p73. p73 is a homologue of p53, and is involved in the apoptotic response to DNA damage. p73 induces apoptosis through transactivation of p53 upregulated modulator of apoptosis (Puma). Puma is a BH3-only protein, and increased puma concentrations cause translocation of pre-formed bax to the mitochondria, initiating apoptosis (Figure 1.4). Cells from mice or drosophila that are deficient in Chk-2 do not undergo apoptosis in response to UV irradiation, demonstrating the importance of the ATR-Chk pathway in DNA-damaged induced apoptosis.

c. PARP-mediated response to single-strand nicks

The DNA damage response pathway that deals with single-strand breaks is not as well understood as the ATR-dependent pathway. However, this pathway is very important in both preventing single strand nicks from becoming more dangerous double-strand breaks and apoptosis in response to DNA damage. The principal molecule involved in the single-strand nick response is PARP. PARP is an enzyme that binds to
Adducts distort the DNA double helix, leading to single-stranded regions. RPA unwinds the double helix further and binds with high affinity to single-stranded DNA. ATRIP-ATR complexes bind to RPA, allowing activation of ATR. Phosphorylated ATR phosphorylates a variety of proteins, among them p73. Phosphorylated p73 induces apoptosis through the mitochondrial pathway.
single-strand breaks in DNA through a zinc finger domain. Upon binding to DNA breaks, PARP becomes enzymatically active, adding poly(ADP-ribose) to itself and surrounding proteins. Poly(ADP-ribose) (PAR) addition allows recognition of the site by DNA polymerases and ligase, which can repair the break. Beyond the role in repair of DNA damage, PARP is involved in the induction of apoptosis in response to DNA damage. Several DNA damaging agents do not induce apoptosis in the absence of PARP. PARP-induced apoptosis occurs as a result of the high energy cost of poly(ADP-ribose)ylation. The process requires high levels of ATP and NAD. Prolonged enzymatic activity of PARP depletes the cell of ATP and NAD. When the concentration of these molecules decrease in the cell, the mitochondrial membrane becomes permeable. Increased permeability of the mitochondrial membrane induces apoptosis by allowing the release of apoptosis inducing factor, cytochrome C and inhibitors of IAPs, and the activation of caspase 9.

d. Evidence for NS1-induced apoptosis

The nonstructural protein NS1 is generally regarded as the best candidate molecule to initiate B19-induced apoptosis. Other possibilities include the viral genome itself and the capsid proteins, but although these have been proposed as inducers of apoptosis, they have not been demonstrated as such. NS1 was initially characterized as a cytotoxic protein because of the difficulties encountered upon attempts to express NS1 in cell culture. Experiments with HeLa cells showed that far fewer antibiotic resistant colonies formed after transfection with plasmids containing NS1 and an antibiotic
Figure 1.4. Mechanism of single-strand nick induced apoptosis. DNA develops single-strand breaks. PARP recognizes and binds to single-strand breaks using a zinc finger domain. Upon binding, PARP is activated and ribosylates surrounding proteins. Ribosylation is energy-intense and leads to a decrease in cellular ATP. Decrease in cellular ATP and NAD levels alters mitochondrial membrane permeability, causing the release of mediators of apoptosis, such as AIF and cytochrome C.
Figure 1.4.
resistance gene than vector plasmids without NS1. NS1, therefore, killed the antibiotic-resistant cells\(^{135}\). Mutational analysis of cloned NS1 indicated that there is a site located in a putative nucleotide triphosphate-binding domain that is necessary for cytotoxicity. Researchers mutated single amino acids in a motif that is conserved in multiple parvoviruses. Cells transfected with mutated NS1 genes formed colonies at a significantly higher frequency than wild type NS1\(^{109}\). This study further confirmed the cytotoxic potential of NS1, and identified a domain necessary for cell killing.

Although these studies indicated that NS1 was killing cells, the mechanism through which cell death was proceeding was unknown. Moffatt et al. clarified the mechanism by demonstrating that NS1 was killing cells of the erythroid lineage through apoptosis\(^{107}\). Using transfected erythroleukemia cells, they demonstrated that NS1-induced cell death was attenuated by caspase 3 inhibitors and blocked by BCL-2 transfection\(^{107}\). The involvement of caspase 3 and BCL-2 in NS1-induced cell death demonstrated that the cells likely die through apoptosis, and that the apoptotic signal is transduced through the mitochondria.

Identification of apoptosis as the mechanism of cell death still left many questions unanswered. There are multiple inducers of apoptosis and multiple pathways through which apoptosis can proceed. Two groups have identified an interaction between NS1 and TNF-\(\alpha\). Fu et al. demonstrated that NS1 was capable of upregulating TNF-\(\alpha\) production through interaction with AP-1 and AP-2 sites in the TNF-\(\alpha\) promoter element\(^{64}\). Sol et al examined the relationship between the death receptors triggered by members of the tumor necrosis factor superfamily, such as fas and TNFR1. They found that NS1 expression correlated with activation of caspases 3, 6, and 8, and that inhibitors
of these caspases were able to decrease cell death. However, although the caspase 8 inhibitor did decrease cell death in this erythroid cell system, it did so to a lesser extent than the other caspase inhibitors. They also found that NS1 sensitized cells to TNF-\(\alpha\) treatment. Normally cells are not sensitive to TNF-\(\alpha\) induced apoptosis unless a protein synthesis inhibitor, such as cycloheximide, is added to the cell culture. However, when erythroid cells are transfected with NS1, TNF-\(\alpha\) alone is able to induce apoptosis. Cells transfected with the vector alone did not show sensitization to TNF-\(\alpha\)^179.

As a result of the difficulties inherent in working with B19, and the cytotoxic nature of NS1, little is known about the mechanisms through which it induces apoptosis. There is scarcely any knowledge of how other parvoviral proteins induce apoptosis. Some studies, however, do illuminate processes that lead to apoptosis in the presence of NS1. The nonstructural proteins of other parvoviruses also induce apoptosis. The rep proteins of AAV, which are homologous to NS1 of B19, can induce or enhance apoptosis when transfected into cells. Transfection of NT-2 or HL-60 cells with AAV rep proteins induces apoptosis in these cells, with approximately 50% of cells undergoing apoptosis within 50 hours^162. AAV rep-induced apoptosis is dependent on caspase 3. Similar caspase-3 dependent apoptosis occurs during infection of glioblastoma cells with H1 parvovirus^132, and feline lymphocytes and epithelial cells^11, upon infection with feline panleukopenia virus^79. The expression of AAV rep proteins blocks the cell cycle, and apoptosis proceeds in the presence or absence of p53^162. In cell lines treated with cadmium or UV irradiation, expression of rep proteins enhances apoptosis^216,217.

NS1 targets a number of cellular factors that influence apoptosis. H1 parvovirus downregulates C-myc expression, an event that also occurs during TNF-\(\alpha\)-induced
apoptosis. Cell lines that resist apoptosis by H1 parvovirus also resist apoptosis by TNF-α. Similarly, cell lines that resist apoptosis by rat parvovirus are also resistant to apoptosis induced by X-ray irradiation. These studies suggest that apoptosis in parvovirus-infected cells occurs through normal cellular apoptotic mechanisms.

Paroviral nonstructural proteins may induce apoptosis by damaging the cellular DNA. The nature of the nicking, DNA binding, and helicase activities of NS1 suggests the distinct possibility that these activities are carried out on the host chromosomes, activating the DNA damage response. This possibility is addressed in the current dissertation, and other groups have found evidence to support the hypothesis that NS1 damages cellular DNA. H1 parvovirus causes low levels of NAD, a state that can be caused by PARP activation in response to DNA damage. Also, nicks appear in cells transfected with NS1 from minute virus of mice, demonstrating that viral genes are capable of damaging the host chromosome.

NS1 is a cytotoxic protein, which causes cells to undergo apoptosis. However, the mechanism by which NS1 initiates apoptosis is completely unknown. DNA damage is a likely cause of NS1-induced apoptosis and it is likely that this apoptosis would involve the nick repair and ATR-mediated pathways. Very little is known about the interactions of NS1 with cellular DNA, or even whether such interactions occur.

**J. Conclusion**

Although unable to replicate in hepatocytes, B19 may cause multiple types of hepatitis, including AFLF. However, due to the ability of B19 to persist for years and the difficulty in working with B19, the relationship between B19 infection and liver disease
is not clear. To understand this relationship better, the effects of B19 infection of liver cells must be understood. Demonstration that B19 has a cytopathic effect on hepatocytes would disprove the hypothesis that B19 is merely a passenger in the cell, and unable to cause deleterious effects.

Although B19 is an extremely small virus, the effects of B19 infection on the cell are multiple and complex. B19 tropism, transcription, replication, and protein expression are regulated in a very specific manner. The expression of B19 proteins, the rate of transcription, and the ability to replicate viral genomic DNA all vary with the cell that is infected. Even in nonpermissive cells, B19 may be able to transcribe genes and modify the activity of the cell. NS1 is preferentially expressed in nonpermissive cells.

Most of the functions of B19 are carried out by NS1. Although NS1 is only one protein, it serves as an initiating factor for DNA replication, an ATPase, a transcription factor, a genome separator and a helicase. NS1, acting alone, is capable of killing cells through apoptosis and blocking the cell cycle. NS1 coordinates the cellular replication and transcription machinery to provide optimal production of virions. NS1 is cytotoxic, so it is difficult to learn about how it accomplishes these many functions. An improved system that controls the cytotoxicity of B19 is needed to study how NS1 interacts with the host cell in vivo. A system that allows inducible expression of a NS1-GFP fusion protein will be discussed in chapter 3.

The many activities of NS1 make determination of the mechanisms of NS1 cytotoxicity complex. NS1 may kill cells by damaging cellular DNA, by modifying cellular transcripts, by inducing the generation of cytokines, or by other unidentified methods. However, considering that the primary role of NS1 is to interact with and
modify DNA, it is probable that the predominant mechanism of NS1-induced cytotoxicity is DNA damage. NS1 could induce DNA damage by nicking, attaching itself to, or unwinding cellular DNA. The DNA repair machinery would recognize these changes through ATR or PARP-mediated pathways, and either repair the NS1-induced damage or initiate apoptosis.
Chapter 2

Parvovirus B19-Induced Apoptosis in Liver Cells
A. Introduction

Parvovirus B19 (B19) is the only parvovirus known to cause disease in humans. B19 is a small, single-stranded DNA virus that is transmitted in blood products or through aerosols and fomite contamination. In patients with chronic hemolytic anemias, such as sickle cell disease or hereditary spherocytosis, B19 infection causes transient aplastic crisis by destroying the erythroid precursor pool.

Transient aplastic crisis presents in up to 1/3 of cases of acute fulminant liver failure (AFLF) that do not involve hepatitis A-E viruses. AFLF is a potentially fatal disease that may occur as a result of hepatic infection, toxic damage, or liver transplantation. Langnas and colleagues demonstrated that a significant number of patients with AFLF-associated with aplastic anemia had parvovirus B19 DNA in the liver detectable by PCR. Karetnyi et al. further correlated AFLF with B19 infection by demonstrating the presence of active B19 infection, as indicated by the presence of viral RNA in liver tissues from patients with AFLF-associated with aplastic anemia. Interestingly, although B19 RNA was detected in liver samples from patients with AFLF, replicative forms of the B19 genome were not, suggesting that transcription occurs without replication of the virus in these tissues. Although B19 cannot replicate in hepatocytes, hepatocytes express globoside, the receptor for B19, and empty capsids will bind to extracts from liver tissue. It is possible, therefore, that B19 can enter hepatocytes and establish a limited infection, thereby damaging the liver.

Other diseases associated with B19 infection also involve cells that are nonpermissive for B19 infection. These diseases include arthropathy, myocarditis, encephalitis, hepatitis, dermatomyositis, and scleroderma. Cooling and
coworkers showed a link between the presence of globoside, the putative B19 receptor, or neutral glycosphingolipids capable of binding B19, in the tissue and the ability of B19 to cause disease in that tissue. The demonstration of the B19 receptor on disease-associated tissues suggests that if the cellular receptor is present, B19 may cause damage even to cells that do not allow for the production of progeny virus.

The only cells that allow B19 to productively replicate are erythroid precursors, including fetal liver, isolated stem and bone marrow cells, and megakaryocytic leukemia cell lines maintained with erythropoietin. Upon infection of erythroid precursors, cell death occurs by apoptosis, and is probably caused by the viral nonstructural protein (NS1), which has been shown to induce apoptosis when transfected into erythroid cells. The mechanisms of cell death, however, including a description of how NS1-induced apoptosis proceeds, have not been described.

Although knowledge of the mechanisms through which B19 affects permissive cells is lacking, still less is understood about the interaction of B19 with cells that do not support viral replication. The block to productive infection in cell types other than erythroid precursors is not well understood, however, aberrant splicing or transcription of the viral capsid proteins has been demonstrated. The block to productive infection, however, does not affect production of NS1. B19 may be able to enter certain cells that express globoside and establish a restricted infection with NS1 as the principal viral product. Broad tropism for viral entry and NS1 production could explain the broad spectrum of disease associated with B19 infection.

To understand the process of B19 infection of nonpermissive cells in general, and hepatocytes specifically, both primary hepatocytes and Hep G2 hepatocellular carcinoma
cells were inoculated with B19. Inoculated cells were analyzed for production of NS1 and induction of apoptosis. Caspase pathways involved in B19-mediated apoptosis were examined using specific caspase inhibitors.

All reports investigating the association of B19 with liver disease agree that B19 DNA can be found in the livers of individuals with AFLF\textsuperscript{57,84,205}. The relevance of the presence of B19 in the liver to a disease state, however, remains to be elucidated. Demonstration of a mechanism through which B19 can damage the liver provides new evidence for an association between B19 and AFLF.

**B. Materials and Methods**

1. **Viruses and cell culture**

   Primary hepatocytes were obtained from BD Gentest (Palo Alto, CA), plated at 50% confluency onto collagen-coated 75 cm\(^2\) flasks and 4-well chamber slides. The donor was an adolescent Caucasian male whose cause of death was anoxia. Primary hepatocytes were maintained in Hepatozyme\textsuperscript{tm} SFM medium with no supplements (Invitrogen, Carlsbad, CA). To investigate the role of cell division in B19 infection of hepatocytes, hepatocyte growth factor (Calbiochem, San Diego, CA) was added to a portion of the slides at a concentration of 40 ng/ml.

   Hep G2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultivated in hepatocyte wash buffer (Invitrogen) with 10% fetal bovine serum (FBS) (Invitrogen). The cells were incubated at 37\(^\circ\)C in a humidified 5% CO\(_2\) incubator. Hep G2 cells were grown in 8-well LabTek II chamber slides (Nalge Nunc International, Rochester, NY) for annexin-V staining and caspase 3 activity
experiments and 75 cm² tissue culture flasks (Fisher, Pittsburgh, PA) for the reverse transcriptase polymerase chain reaction (RT-PCR) experiments.

Viremic serum from a patient with aplastic crisis was used as a B19 inoculate. The viral titer of this serum was previously determined by PCR to contain $1 \times 10^{12}$ viral genomes/ml.

2. Inoculation with B19

Primary hepatocytes were inoculated with 1000 viral genome equivalents/cell in Hepatozyme™ SFM serum free medium. To improve detection of transcription and replication of B19, Hep G2 cells were synchronized prior to inoculation by adding hydroxyurea to the cell culture medium at a final concentration of 2 mM. Cells were incubated with hydroxyurea for 40 hours, and then washed 4 times with wash buffer without serum. Synchronization was verified by staining cell nuclei with propidium iodide followed by DNA quantification using flow cytometry.

After washing, the cells were inoculated with viremic serum. To determine the optimal dose of B19, the amount of apoptosis in response to increasing viral dose was investigated with 10, 100, and 1000 viral particles per cell. Due to restricted supplies of B19, 1000 viral particles per cell was the maximum ratio that could be used. In subsequent experiments, 1000 viral particles/cell were added to synchronized cells in Opti-Mem™ (Invitrogen) serum free medium. After 18 hours of incubation at 37°C, the cells were washed and maintained in hepatocyte wash medium supplemented with 10% FBS.
3. RT-PCR

All steps involving RNA were performed in RNase-free plastic using RNase-free solutions. Hep-G2 cells were grown to a concentration of $1 \times 10^7$ cells per flask and synchronized, then inoculated with $1 \times 10^{10}$ (10 µl serum) viral particles in 5mL medium. Primary hepatocytes were inoculated 2 days after isolation with $1 \times 10^{10}$ viral particles per flask as described for Hep G2 cells. Three days post-inoculation, cells were trypsinized, centrifuged in medium at 200xg for 5 minutes and the medium removed. Cells were then lysed in 2 ml Trizol™ reagent (Invitrogen). Total RNA was extracted following the manufacturer’s directions. RNA was resuspended in 500 µL RNase-free water (Invitrogen) and stored at -85°C until used.

To rule out the possibility of false-positive signals due to DNA contamination, isolated RNA was treated with 10 units RNase free DNase (Roche, Indianapolis, IN) in RQ1 DNase buffer (Promega, Madison, WI) for 1 hr at 37°C. One µg of RNA isolated from infected cells was incubated with 10 units DNase-free RNase (Roche) 1 hr at 37°C. RT-PCR was performed on 1 µg (pretreatment mass) of RNA from each condition using the Superscript™ One Step RT-PCR with Platinum Taq™ system (Invitrogen). The PCR reaction was prepared by adding 1 µg of extracted RNA to 47 µl of reaction buffer containing 0.2 µM of the appropriate primers, and 1 µl of RT-Taq mix. To further ensure that a positive signal was not the result of DNA contamination, a PCR reaction mix was prepared as above with the exception that one µg of DNase treated RNA was used and Platinum-Taq™ polymerase replaced the RT-Taq mix. For a positive PCR control, a reaction mix was prepared as above with 1 µl viremic serum replacing the RNA in one tube.
For detection of NS1, the upstream primer consisted of the sequence 5'CAGTGGAAGTTTTCAAATTCAAAGT3' and the downstream primer 5'ATGGCTTTTGCAGCTTC3'. For the detection of VP1/VP2, the primer sequences were upstream 5'ATACTCAACCCCATGGAGA3' and downstream 5'TTCTGATATGGTTACAGTT3' as previously described. All samples were incubated at 45°C for 30 minutes, denatured at 94°C for 2 minutes, then subjected to 40 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for NS1 detection and 52°C for VP1/VP2 detection for 2 minutes, and elongation at 72°C for 1 minute. During the last 14 cycles the elongation time was extended by 15 seconds per cycle. PCR products were analyzed by electrophoresis through a 1.2% agarose submarine electrophoresis gel and staining with ethidium bromide.

4. Immunofluorescence

Immunofluorescence was used to detect NS1 protein, confirming viral infection. Hep G2 cells were grown on glass coverslips. The cells were synchronized and infected with 1000 viral particles per ml. At 0, 8, 24, 48, 72, and 96 hours post-infection, coverslips were removed from the wells and the cells fixed in 4% paraformaldehyde. Cells were permeablized with 0.2% Triton-X 100 (Fisher) in PBS for 10 minutes, then washed. After blocking with 10% goat serum for 1 hour, the anti-NS1 monoclonal antibody ParC-NS1 (a generous gift from Kazuo Sugamura, Tohoku University) was diluted 1:200 in PBS and added to the coverslips. An isotype control antibody for mouse IgG1 (Oncogene) was diluted 1:200 and incubated with infected cells as a negative control. Coverslips were washed 3x with PBS, and a 1:500 dilution of chicken anti-
mouse IgG conjugated to alexa-fluor 488 (Molecular Probes, Eugene, OR) was added to the coverslips. The coverslips were incubated with the secondary antibody for 1 hour, and then washed 3x with PBS. To enhance detection of NS1, a 1:500 dilution of goat anti-chicken Ig conjugated to alexa-fluor 488 in PBS was incubated with the coverslips for 1 hour, and then washed 3x with PBS. Fluorescence was viewed utilizing a standard FITC filter set with a 450-490 nm excitation filter and a 515 nm longpass barrier filter. The antibody dilutions used were determined through staining of infected cells with dilutions from 10-fold to 1000-fold, and selecting the optimal antibody concentration.

5. Western blotting and PARP cleavage

Apoptosis was investigated using a western blot to detect PARP cleavage. Hep G2 cells were grown in 6-well plates and infected with 1, 10, 100, or 1000 viral particles/ml B19. Cells were lysed with 1%w/v SDS, 4M urea, and 0.7M 2-mercaptoethanol. As positive controls, Hep G2 cells were treated with 14 ng/ml TNF-α, and lysed after 8 or 24 hours of TNF-α treatment. Lysates were boiled 5 minutes, then electrophoresed through a 4-15% polyacrylamide gel. Proteins were transferred to nitrocellulose for 1 hour at 100 volts in a Trans-Blot transfer apparatus (Bio-Rad). PARP was detected by incubating the membranes with a 1:5000 dilution of a monoclonal anti-PARP antibody (BD Pharmingen, San Diego, CA) in TBS-Tween. The membrane was washed 6x with TBS-Tween, and a 1:10,000 dilution of anti-mouse IgG conjugated to horseradish peroxidase (Amersham) was incubated for one hour with the membrane. After 6 washes, PARP was visualized by ECL+™ chemiluminescence (Amersham).
6. Annexin-V staining

Virus-containing serum was diluted 1:1000 in serum-free OPTI-MEM® (Invitrogen) medium and incubated with either anti-B19 neutralizing antibody (NCL-PARVO, Novocastra Laboratories, Newcastle upon Tyne, UK) or irrelevant isotype control (mouse IgG1, Pharmingen) at a 1:100 dilution for 60 minutes at 4°C. For UV-treated inoculates, virus-containing serum was diluted 1:1000 and exposed to 2 joules/cm² UV irradiation in an Ultra-Lum UVC 508 UV crosslinker. Hep G2 cells grown in LabTek II™ chamber slides to a density of 2 x 10⁵ cells/cm² were synchronized with hydroxyurea 2 mM for 40 hours. The cells were washed 4x with serum free hepatocyte wash medium and inoculated with 200 µL/well of NCL-PARVO-treated diluted serum, isotype control-treated diluted serum, non-viremic diluted serum, or UV-irradiated viremic serum, for a final ratio of 1000 viral particles/cell as described above.

To determine the time course of apoptosis, Hep G2 cells were infected and apoptosis was quantified by annexin-V staining at one, two, three, and four days post-infection. Subsequent annexin-V staining and caspase activity experiments were performed three days post-infection.

After inoculation with B19-containing or control serum, all cells were incubated for 3 days at 37°C in 5% CO₂. Growth medium was removed and 500 µL wash medium, 10 µL media binding reagent, and 1.25 µL annexin-V FITC (Oncogene, Boston MA) were added to each well. The slides were incubated for 15 minutes at room temperature, then the medium was aspirated, growth chambers removed from the slides, and the cells covered in binding buffer. Slides were immediately analyzed by fluorescence microscopy using a standard FITC filter set with a 450-490 nm excitation filter and a 515
nm longpass barrier filter. Green fluorescing cells were counted and compared to total cells to determine the percentage of annexin-V positive cells. Duplicate wells were averaged with three fields per well counted.

7. Caspase 3 activity

Hep G2 cells were grown in supplemented hepatocyte wash buffer, synchronized with hydroxyurea, and inoculated at a ratio of 1000 viral particles/cell in 8 well LabTek II slides as described above. Three days post-infection the slides were washed and the media and chambers removed. Slides were covered with PhiPhiLux G2D2™ caspase 3 fluorescent substrate (Oncogene) and incubated at 37°C for 45 min. The slides were then washed 4x with PBS, and examined immediately by fluorescence microscopy using a 528-553 nm excitation filter and a 600-660 nm barrier filter. Bright red fluorescing cells were counted and divided by the total cells present in order to determine the percentage of cells positive for caspase 3 activity. Duplicate wells were averaged for each experiment, with at least three fields counted per well. Three independent experiments were performed.

8. Caspase inhibition

Cells were inoculated as for annexin-V staining, with the exception that the cell-permeable caspase 3 inhibitor DEVD-FMK, caspase inhibitor WEHD-FMK, caspase 9 inhibitor LEHD-FMK, or caspase 8 inhibitor IETD-FMK (Oncogene) was added to a final concentration of 10 µM 6 hours before addition of the inoculate. Slides were also prepared with caspase inhibitors added to the medium, but not inoculated, in order to
control for potential effects of the caspase inhibitors. Slides were incubated 3 days and analyzed by annexin-V staining as described above.

8. Statistical analysis

Significance was calculated using the student's $t$ test to evaluate the null hypothesis that the treatment under consideration did not affect apoptosis or caspase activity. $p$ values of less than 0.05 were considered significant.

C. Results

1. NS1 is expressed in Hep G2 cells in restricted infection

Establishment of B19 as a causal agent of AFLF requires proof that B19 is capable of infecting hepatocytes. B19 is a DNA virus, so RNA transcripts of B19 genes are only found after transcription of viral genes by the host cell. To test the hypothesis that B19 is able to establish a limited infection of hepatocytes with the production of NS1, RT-PCR analysis was performed on RNA isolated from primary hepatocytes and Hep G2 cells inoculated with B19 viremic serum. In order to increase the amount of viral transcription, the cells were synchronized at the beginning of S-phase with 2 mM hydroxyurea. Synchronization led to an accumulation of cells at the G1/S border, as demonstrated by DNA content. Of the unsynchronized cells, 11.5% were in S phase. After synchronization, 37% of cells were in S phase, at 7 hours 88% were in S phase, and at 22 hours 38% were in S-phase. After 22 hours, most of the cells had moved through mitosis (Figure 2.1).
Figure 2.1. Synchronization of Hep G2 cells. Hep G2 cells were synchronized by the addition of 2 mM hydroxyurea to the cell culture medium for 40 hours. Cells were harvested that were unsynchronized, at the time of release from synchronization, 7 hours after washing, and 22 hours after washing. Cells were treated with trypsin and 1% (v/v) Triton-X 100 and stained with propidium iodide. Cell cycle was determined by measurement of DNA content by flow cytometric determination of propidium iodide fluorescence.
Figure 2.1
Synchronized Hep G2 cells were inoculated with B19, and RNA was isolated 3 days after infection. In order to demonstrate that any RT-PCR product found was truly RNA, and not contaminating DNA, the RNA was divided into 1 µg aliquots and treated with either DNase or RNase. A 1 µg aliquot was also used for a PCR reaction identical to the RT-PCR reaction, with the exception that the reverse transcriptase was omitted. RT-PCR was performed using primers specific for either NS1 or the common region of VP1/VP2.

A specific 403 bp NS1 amplification product was present in the lanes representing the DNase-treated and positive control conditions, demonstrating the presence of RNA transcripts of the NS1 gene. No PCR products were detected in either the RNase-treated or PCR-only lanes, confirming that the template for the PCR reaction was RNA, and not contaminating DNA. There was no detectable PCR product when the primers for VP1/VP2 were used (Figure 2.2), although the primers were functional as demonstrated by the presence of a specific band in the positive control condition. The presence of viral RNA for NS1 indicates that despite being unable to replicate in Hep G2 cells, B19 is able to enter the cells and utilize cellular machinery to produce transcripts for NS1. The absence of VP1/VP2 transcripts demonstrated that infection is restricted, with no production of the capsid proteins that would be necessary to generate progeny virions.

Functionality of the RNA transcripts of NS1 was demonstrated by immunofluorescence staining for NS1 protein. Immunofluorescence demonstrated bright punctate nuclear staining of 36% of infected cells by 48 hours post-infection. Nuclear localization was visualized by Hoescht 33342 staining of DNA. Light micrographs were obtained using a namarski optics package. No specific staining was seen in isotype control stained cells or uninfected cells (Figure 2.3). The presence of NS1 protein in
Figure 2.2. RT-PCR analysis of infected hepatocytes. Hep G2 cells or primary hepatocytes were inoculated with B19, and RNA was extracted 3 days post inoculation. Aliquots were treated with DNase or RNase, and RT-PCR was performed on 1 μg of RNA with primers specific for NS1 or VP1/VP2. A. RNA template from Hep G2 cells with primers for NS1. B. RNA template from Hep G2 cells with primers for VP1/VP2. C. RNA template from primary hepatocytes with primers for NS1. D. RNA template from primary hepatocytes with primers for VP1/VP2. DNA contamination was excluded by the absence of a band in the RNase treated samples and the PCR only samples
<table>
<thead>
<tr>
<th>NS1</th>
<th>VP1/VP2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td>Hep G2</td>
</tr>
<tr>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td>Primary Hepatocytes</td>
</tr>
</tbody>
</table>

**Figure 2.2**
Figure 2.3. Immunofluorescence of NS1 protein. Hep G2 cells were infected with B19. 48 hours post-infection, cells were stained with anti-NS1 monoclonal antibody or isotype control antibody. Secondary staining was performed with alexa-fluor 488-conjugated anti-mouse IgG (green). NS1 appears as punctuate nuclear fluorescence in the infected cells (green), but not in the uninfected cells or on the isotype control-treated slides.
Figure 2.3

<table>
<thead>
<tr>
<th>B19 inoculated</th>
<th>Uninoculated</th>
<th>Isotype control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Merge</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hoechst 33342</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>NS1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nomarski</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
infected cells indicates that translation of NS1 transcripts occurs in Hep G2 cells, and therefore the transcripts seen in the RT-PCR assays are functional. The presence of NS1 protein suggests that NS1 may be the causal agent in cellular effects of B19 infection.

2. B19 infection induces apoptosis in liver cells

After confirming the capacity of B19 to establish infection of liver cells, the ability of B19 infection to induce apoptosis was studied. To determine whether B19 infection induced apoptosis, primary hepatocytes and Hep G2 cells were inoculated with 10, 100, or 1000 viral particles/cell, incubated for 3 days, and then lysed and analyzed for PARP cleavage by immunoblotting. PARP, a DNA repair enzyme, is cleaved by caspase 3 during late stages of apoptosis. There were detectable levels of cleaved PARP in the lysates from infected cells. The amount of cleaved PARP increased with increasing viral dose (Figure 2.4). Cleaved PARP indicates that apoptosis is occurring in cultures infected with B19.

The apoptosis that was observed in the PARP cleavage assays was confirmed and quantified using annexin-V-FITC staining. Annexin-V binds specifically to phosphatidylserine (PS). PS is normally found only on the inner leaflet of the cell membrane, but during the early stages of apoptosis, this polarity is lost and PS can be detected on the external surface of the cell. In a dose-response experiment utilizing dilutions of virus from 1 to 1000 viral particles per cell, the highest apoptotic response to B19 was seen at a ratio of 1000 particles per cell, similar to what was seen in the PARP cleavage experiments (Figure 2.5).
Figure 2.4. B19 infection of Hep G2 Cells induces apoptosis as demonstrated by PARP cleavage. Hep G2 cells were infected with B19. Three days post-infection the cells were lysed. Lysates were electrophoresed through a 4-15% polyacrylamide gel in the presence of 1% SDS. Protein was transferred to nitrocellulose by electroblotting. Immunoblotting was performed with anti-PARP antibody.
Figure 2.4
Figure 2.5. B19-induced apoptosis is viral dose-dependent. Hep G2 cells were incubated with 10, 100, or 1000 viral particles per ml. Increasing virus-to-cell ratios leads to increased apoptosis as measured by annexin V staining in Hep G2 cells. n=3 experiments, with error bars demonstrating standard deviation.
**Figure 2.6. B19 infection induces apoptosis in Hep G2 cells.** Hep G2 cells were synchronized and infected with 1000 B19 particles/cell. Apoptosis was analyzed by annexin V-FITC staining and fluorescence microscopy. **A.** Micrographs demonstrating annexin V-FITC labeling of apoptotic B19 infected cells (green). **B.** Quantification of apoptosis due to B19 infection. Inoculation of Hep G2 cells with B19 induces significantly more apoptosis than inoculation with nonviremic serum ($p<0.005$), antibody neutralized serum (NCL-PARVO) ($p<0.005$), or B19-containing serum that has been inactivated by UV irradiation ($p<0.04$). The means of three independent experiments, with two wells averaged per experiment, are shown. Error bars represent standard deviation of the means of the experiments.
Figure 2. 6 B
Although a multiplicity of infection of 1000 seems high compared to many viral studies, which use a lower proportion of virus particles to cells, this amount of B19 was probably necessary because B19 is not able to replicate in liver cells. Therefore, the primary inoculate is all of the virus that will be present in the cultures. In contrast, for infection of bone marrow cultures with B19, a lower moi can be used because of viral amplification through subsequent virus production. Furthermore, 1000 viral genome equivalents per cell is not strictly a moi, but rather a quantification of viral DNA. Due to the scarcity of B19 sources, it is necessary to utilize viremic serum as an inoculate, and serum contains inhibitors to B19 binding\(^{19}\), reducing the levels of infectious virions. The concentration of B19 virus used in these experiments is physiologic in that viremic serum contains 1000-fold more virions per milliliter than the inoculate.

After discovering that the maximum levels of apoptosis were seen with 1000 vp/cell, subsequent experiments utilized 1000 vp/cell inoculates. As controls, cells were mock inoculated with non-viremic serum (Sigma), NCL-PARVO-neutralized viremic serum, or UV-irradiated viremic serum. NCL-PARVO has been shown to eliminate B19 activity\(^{154}\). Similarly, UV irradiation is an effective way of eliminating contaminating B19 from blood supplies\(^{187}\), and functions by inhibiting transcription of B19 genes\(^{110}\). UV-irradiated serum is a control not only for serum factors that may affect cells and are found in the B19 inoculate, but also for the effects of viral capsid protein and genomic DNA.

B19-and mock-inoculated cells were stained with annexin-V to detect apoptosis. Examination of stained cells demonstrated that approximately three-fold more cells
underwent apoptosis in the inoculated cultures than in the mock-inoculated cultures. In Hep G2 cultures, 28% of cells in inoculated versus 10% of cells in mock-inoculated cultures underwent apoptosis. Pretreatment of viral inoculates with NCL-PARVO or UV irradiation of the inoculate reduced apoptosis in Hep G2 cells to 7% and 12% of cells, respectively (Figure 2.5). Similarly, approximately three-fold more primary hepatocytes underwent apoptosis when inoculated with B19, 24%, versus inoculation with UV irradiated B19, 7.6% (Figure 2.7). To determine the time course of apoptosis, infected cells were stained with annexin-V-FITC at 1, 2, 3, and 4 days post-infection. Apoptosis was detectable on day 1, and increased on days 2 and 3, after which it declined sharply (Figure 2.8). The decrease in apoptosis after day 3 is indicative that the virus is no longer active in these cultures, reaffirming the assertion that B19 does not replicate in Hep G2 cells.

After discovering that B19 induces apoptosis in both primary hepatocytes and Hep G2 cells, the effects of hepatocyte growth factor (HGF) on B19-induced apoptosis was examined. The paradigm of parvoviral growth maintains that parvoviruses can only grow in dividing cells, and that all parvoviral functions are more active in S-phase. To induce the normally quiescent primary hepatocytes to divide, they were incubated with 10 ng/ml HGF. Although the addition of HGF to the cell culture medium did not lead to a noticeable effect on cell division, those cells treated with HGF were much more resistant to apoptosis resulting from B19 infection than those without HGF, contradicting the expected result (Figure 2.9).

It is surprising that the anti-apoptotic activity of the hepatocyte growth factor is sufficient to significantly decrease the amount of apoptosis even though addition of HGF
Figure 2.7. B19 infection induces apoptosis in primary hepatocytes. Primary hepatocytes were plated and infected with 1000 virus particles/cell. Apoptosis was analyzed by annexin-V staining and fluorescence microscopy. Two wells were counted on each of three different slides. Error bars represent standard deviation. A. Micrographs demonstrating apoptosis of infected cells. Apoptotic cells stain green. B. Quantification of apoptosis due to B19 infection. B19 infection induces significantly more apoptosis than UV-inactivated B19 inoculate \( p<0.008 \).
Figure 2.8. Time course of B19-induced apoptosis of Hep G2 cells. Hep G2 cells were synchronized and infected with 1000 viral particles/cell. Apoptosis was assayed by annexin-V staining at 1, 2, and 3 days post-infection. n=3 independent experiments.
Figure 2.8
Figure 2.9. Hepatocyte growth factor abrogates B19-induced apoptosis in primary hepatocytes. Primary hepatocytes were infected with 1000 viral particles per cell. Hepatocyte growth factor was added to ½ of the cultures. Three days post-infection, apoptosis was assayed by annexin-V staining. Addition of hepatocyte growth factor to the growth medium reduces apoptosis of infected cells from 24% to 10.4% ($p<0.005$). Three separate slides were analyzed for each condition. Background apoptosis was also reduced upon addition of HGF as seen in the culture inoculated with UV-irradiated B19.
possibly increased cell division. The decrease in apoptosis in HGF-treated hepatocytes demonstrates that the apoptosis induced by B19 infection may not be completely dependent on cell division. It is likely that an apoptotic pathway that is influenced by HGF plays a greater role in B19-induced apoptosis of hepatocytes than the ability of the cells to divide. The observed decrease in apoptosis upon addition of HGF may be explained by the findings of Fan et al. who demonstrated that HGF increases production of Bcl-XL, a mitochondrial anti-apoptotic factor, and that HGF treatment increases the efficiency of DNA repair. If B19-induced apoptosis utilizes the mitochondrial pathway to induce apoptosis, treatment of the infected cells with hepatocyte growth factor would be expected to inhibit apoptosis. The finding that a decrease in apoptosis is found in HGF-treated cells suggests the mitochondrial pathway as the apoptotic pathway induced by B19 infection of hepatocytes.

In addition to providing clues about the pathway that B19 infection uses to induce apoptosis, the finding of decreased apoptosis upon addition of HGF to B19-infected cells suggests that DNA damage may be the initiating factor in B19-induced apoptosis. HGF is known to increase the efficiency of DNA repair while inhibiting apoptosis. Therefore, an agent which damages DNA would likely have less of an apoptosis-inducing effect on cells treated with HGF. B19 may cause apoptosis by damaging DNA, an activity which would be abrogated upon the addition of HGF. The hypothesis that B19 induces DNA damage is consistent with the observed decrease in apoptosis upon addition of HGF to B19-infected primary hepatocytes.
3. B19-induced apoptosis requires caspases 3 and 9, but not caspase 8

After obtaining suggestive data as to which apoptotic pathways are utilized by B19, the specific caspases used in the process of B19-induced apoptosis were investigated. Apoptosis is carried out through the activation of a family of cysteine proteases called caspases. Caspase 3 is a downstream caspase that cleaves nuclear proteins and activates other caspase family members. Caspase 3 activation is a marker for apoptosis. Caspase 3 has been implicated in apoptosis induced by B19 in permissive cells, and in apoptosis induced by the Rep 78 protein of adeno-associated virus (AAV), another human parvovirus.\[^{162}\]

Caspase 3 activation was examined in Hep G2 cells using the cell-permeable fluorescent caspase 3 substrate PhiPhiLux G$_2$D$_2$. If caspase 3 is active in the cell, PhiPhiLux G$_2$D$_2$ is cleaved and trapped intracellularly and can be detected by fluorescence microscopy. In inoculated Hep G2 cells, over twice as many inoculated cells were positive for caspase 3 activity as were mock-inoculated cells, 44.6% of inoculated cells versus 20.6% of uninoculated cells (Figure 2.10). Caspase 3 activation in B19-infected cells is another indicator that the cells are undergoing death through apoptosis.

Incomplete removal of uncleaved PhiPhiLux G$_2$D$_2$ despite washing leads to higher background for mock-inoculated cells in caspase 3 assays (Figure 2.5B, 20.6%) versus annexin-V staining assays (Figure 2.9, 10%). Annexin-V staining was used to assay for apoptosis detection because of its greater specificity for apoptosis, but PhiPhiLux substrate staining to demonstrate caspase 3 activity.
Figure 2.10. Caspase 3 is activated by B19 infection. Caspase 3 activity was assayed by PhiPhiLux G2D2 staining. (A) Cells with caspase 3 activity stain red. (B) Cells incubated with viremic serum neutralized with NCL-PARVO antibody show a lower percentage of cells with active caspase 3 than cells incubated with viremic serum and an irrelevant isotype control antibody (p=0.006). Values represent means of 3 independent experiments, with 2 wells counted per experiment. Error bars represent standard
Figure 2.10 A
To investigate whether caspase 3 activation was required for B19-induced apoptosis, Caspase 3 was inhibited with DEVD-FMK, a cell-permeable selective caspase 3 inhibitor. Apoptosis was detected in B19-inoculated Hep G2 cells using annexin-V staining. DEVD-FMK blocked apoptosis by B19, returning the number of annexin-V positive cells to background levels, 5.6% in Hep G2 cells and 7.3% in primary hepatocytes (Figure 2.11). After finding that caspase 3 was active in B19-inoculated cells, and that caspase 3 activation was necessary for B19–induced apoptosis, the involvement of other caspases in B19-induced apoptosis were examined.

Caspase 8 is one of the primary caspases activated by TNF-α, and transduces extracellular signals for apoptosis. The pathways that induce apoptosis in response to endogenous stimuli, for example in response to DNA damage or growth factor withdrawal, are transduced through the mitochondria, and utilize caspase 9. Previous studies have suggested that B19 infection can sensitize erythroid cells to TNF-α-induced apoptosis\textsuperscript{179}, a pathway that would proceed through caspase 8. Caspase 8 directly activates caspase 3, which further transduces the apoptotic cascade leading to cell death\textsuperscript{185}.

The literature indicating that apoptosis induced by B19 is a result of TNF-α suggested that caspase 8 would be essential for apoptosis in the B19 infected hepatocytes, while the data from the HGF experiment suggested that caspase 9 would be the predominant caspase. To discern the specific apoptotic pathways utilized during B19 infection, Hep G2 and primary liver cells were inoculated with B19, and specific caspases were inhibited with pharmacologic inhibitors. Caspase 8 was inhibited with IETD-FMK, an irreversible cell permeable selective caspase 8 inhibitor. Caspase 9 was
Figure 2.11. Apoptosis induced by B19 in Hep G2 cells is dependent on caspase 3 and 9, activity, but not caspase 8. Hep G2 cells were inoculated with 1000 viral particles per cell of B19-containing serum or mock infected with an equivalent amount of non-viremic serum in the presence of the caspase inhibitors IETD-FMK (inhibits caspase 8 and granzyme B), DEVD-FMK (inhibits caspase 3, and to a lesser extent 6, 7, 9, and 10), WEHD-FMK (inhibits caspases 1, 4, and 5), or LEHD-FMK (inhibits caspases 9 and to a lesser extent 1, 4, and 5). Caspase inhibition with DEVD-FMK and LEHD-FMK significantly decreased apoptosis in infected Hep G2 cells ($p<0.001$, and $p<0.001$, respectively), while WEHD-FMK and IETD-FMK showed no significant decrease in apoptosis as measured by annexin-V staining. Three independent experiments were performed. Error bars represent standard deviation.
Figure 2.11
Figure 2.12. Inhibition of caspases 3 and 9 abrogates apoptosis in primary hepatocytes. 6 hours before infection, primary hepatocytes were incubated with the specific caspase inhibitors DEVD-FMK (caspase 3), IETD-FMK (caspse 8), LEHD-FMK (caspase 9), or WEHD-FMK (caspase 5). Cells were infected with 1000 viral particles/cell. Three days post-infection, apoptosis was assessed using annexin V staining. DEVD-FMK and LEHD-FMK inhibited apoptosis to a significant extent, returning the levels of apoptosis to background ($p<0.002$ and $p<0.01$, respectively). In the absence of infection the inhibitors did not demonstrate any effects on apoptosis.
Figure 2.12
inhibited with LEHD-FMK. Cultures were treated with the caspase inhibitor WEHD-FMK to control for the minor inhibition of caspases 4 and 5 by LEHD-FMK. WEHD-FMK inhibits caspasess 1, 4, and 5, but not 9\textsuperscript{115}. Hep G2 cells treated with WEHD-FMK or IETD-FMK demonstrated no decrease in apoptosis, while in those treated with DEVD-FMK or LEHD-FMK there was a significant decrease in apoptosis with a return to background levels (Figure 2.11).

The experiments examining the necessity for specific caspase activity in B19-infected Hep G2 cells were repeated in primary hepatocytes. In cultures of B19-infected primary hepatocytes, there was a significantly large decrease in apoptosis, with a return to background levels of apoptosis of after treatment with the caspase 3 inhibitor DEVD-FMK or the caspase 9 inhibitor LEHD-FMK. No decrease in apoptosis was seen upon incubation with the caspase 8 inhibitor IETD-FMK or the caspase 1, 4, and 5 inhibitor WEHD-FMK. The caspase 8 inhibitor IETD-FMK was able to abrogate apoptosis in Hep G2 cells treated with an anti-Fas antibody, demonstrating that the lack of inhibition in these studies is not due to lack of activity of the inhibitor. In the absence of infection, none of the caspase inhibitors markedly affected the cells (Figure 2.12).

These findings indicate that in hepatocytes, B19 induces apoptosis through a caspase 9-dependent, caspase 8-independent pathway. The lack of involvement of caspase 8 suggests that the TNF-receptor family is not necessary for apoptosis in B19-inoculated cells, and that apoptosis proceeds through an intrinsic pathway, possibly as a result of DNA damage or an as yet undiscovered process.
D. Conclusion

Although unable to productively replicate in hepatocytes, B19 is able to enter Hep G2 cells and hepatocytes and generate NS1 protein. B19 infection of hepatocytes produces NS1, although there is a block to the generation or stability of the transcripts for VP1 and VP2. The ability of B19 to establish limited infection in hepatocytes demonstrates that cells other than erythroid precursors can fulfill the initial requirements, such as receptor binding and internalization, for parvovirus B19 infection. Therefore, availability of the receptor for B19 is not the only determinant of permissiveness for B19 replication. The ability of B19 to infect ex vivo primary hepatocytes demonstrates that B19 infection of hepatocytes is physiologically relevant to normal cells, and is not an artifact of the transformed state of the model cell line Hep G2.

Instead of being an innocent bystander in liver tissue, B19 infection of both primary and transformed hepatocytes causes cell death by apoptosis in one third of the inoculated cells. The apoptotic nature of the cell death was confirmed by three separate apoptosis assays, PARP cleavage, annexin-V staining, and caspase 3 activation. Hepatocellular death through apoptosis is consistent with the pathology demonstrated in cases of AFLF-AA, in which noninflammatory disappearance of hepatocytes is the primary finding. Apoptosis, in contrast to necrosis, causes cells to die without leading to inflammation in most cases.

Expression of NS1 is likely to be the cause of the apoptosis observed in B19-infected hepatocytes, since irradiated virions do not have any affect on the liver cells, and are unable to transcribe their genes. The hypothesis that NS1 is the causal molecule of
B19-induced apoptosis is strengthened by the finding that transcription of NS1, but not the structural proteins, was observed in B19 infected cells.

Investigation into the caspase pathways involved in B19-induced apoptosis further suggests NS1 as the molecule which induces apoptosis. Apoptosis of hepatocytes in response to B19 infection proceeds through a caspase 9-dependent pathway, indicating that an intrinsic signal, such as DNA damage, hypoxia, or growth factor withdrawal, provides the apoptotic stimuli, rather than an extrinsic signal, such as TNF-α stimulation. NS1 has the potential to cause this initiating signal through DNA-modifying activities. If NS1 damages cellular DNA in the same manner as NS1 interacts with viral genomes, it would cause DNA damage in the form of DNA nicks and bulky adducts. Both of these types of DNA lesions can initiate pathways leading to apoptosis. DNA damage-induced apoptosis proceeds through the mitochondrial caspase 9-dependent pathway, which is consistent with the apoptotic pathway utilized by B19 infection.

The data obtained through the experiments presented in this chapter demonstrate that B19 is not innocuous in liver cells. B19 can infect hepatocytes, and B19 infection of liver causes apoptosis. B19 infection has the potential to effect significant damage to the liver in vivo. This work defines a mechanism through which B19 infection could lead to acute fulminant liver failure and other manifestations of B19-associated hepatitis. Furthermore, the data demonstrate an intrinsic pathway for B19-induced apoptosis and suggest NS1 as the viral factor responsible for apoptosis.
Chapter 3

NS1-Induced Apoptosis of Liver Cells
A. Introduction

In the preceding chapter it was demonstrated that B19 infection of liver cells causes those cells to undergo apoptosis, and the hypothesis that the apoptosis was due to the activity of the NS1 protein of B19 was presented. NS1 is a multifunctional protein with NTP binding, helicase, nickase, and transcription factor activities. NS1 has been shown to be cytotoxic when transfected into erythroid cells\textsuperscript{107}. However, the mechanisms through which NS1 induces apoptosis are not well understood.

This study utilized cloned NS1 under the control of an inducible promoter to examine the mechanisms of NS1-induced apoptosis in the absence of other viral components. The NS1 DNA sequence was fused to that of an enhanced mutant of green fluorescent protein (eGFP) from \textit{Aqueora victoria}. NS1 is toxic when expressed, so standard expression vectors were not sufficient for these experiments. The vector system that was created consists of a protein expression vector and a second plasmid, pVGRXR, which encodes the ecdysone receptor construct necessary to induce transcription from the expression vector. The combination of an inducible vector that expresses NS1 fused to eGFP allows the determination of the cellular effects of NS1 on a single cell basis at a defined time point.

This system overcomes several problems usually encountered when investigating parvoviral nonstructural proteins. First, regulated expression of the NS1 fusion protein enables examination of the effects of NS1 without interfering effects from transfection reagents. Secondly, many cell types, including liver cells, are very difficult to transfec. Bulk culture studies to examine the effects of a protein do not provide adequate data when only 1-2\% of the cells in a culture express the protein. Through visualization of the
eGFP tag on NS1, individually transfected cells were identified and examined. Transfection with the parental vector, which expresses eGFP, provides an ideal control for the effects of transfection and foreign protein expression, while at the same time controlling for the effects of GFP in the cell.

The eGFP/NS1 vector was utilized to investigate two likely mechanisms for NS1-induced apoptosis. The transcriptional activating properties of B19 have been associated with increased production of TNF-α by B19-infected erythroid precursors. B19-infected erythroid precursors are also sensitized to apoptosis induced by TNF-α. It is possible that NS1 activity induces apoptosis through the TNF-α pathway. The second potential mechanism for NS1 induction of apoptosis is through the DNA damaging activities of NS1. If the DNA modifying activities of NS1 that control viral replication also modified cellular DNA, a DNA damage response cascade would be initiated that could lead to apoptosis. In these studies, the ability of NS1 to damage DNA was examined directly, and the involvement of DNA repair pathways in NS1-induced apoptosis was analyzed.

**B. Materials and Methods**

1. **Cloning of NS1**

   NS1 was cloned from B19 viremic serum using PCR. The primers used were upper 5' GTGAGCTAACTAACAGGTATTTATACCCCGAGCTCGAACATCTCTTAACA3'; lower 5' TAACCTTTTCATAAAATTCACAAATGCTGCGAGCTCGCTTTAGC 3'. The amplicon incorporates nucleotides 391 to 2535 of the B19 genome, and adds *sac I* restriction enzyme cut sites at nt 421 and 2498. The italics in the primer sequences above
indicate the *sac* I cut sites. The cloned sequence includes the entire protein-coding region of the NS1 gene.

Two-temperature PCR was performed using B19 viremic serum as a template for 28 cycles of denaturation 95°C for 15 seconds, and annealing/extension at 59.5°C for 2 minutes 30 seconds. Subsequently, 14 additional cycles were performed in which the annealing/extension step was extended 15 seconds/cycle. The amplicon was analyzed on a 0.5% agarose gel stained with ethidium bromide and the band at 2144 bp was cut out with a scalpel. The DNA was purified from the agarose gel using the Qiaquick gel extraction kit (Qiagen), and digested with 10 units *sac* I (Promega, Madison, WI) for 24 hours.

The inducible eGFP/NS1 fusion protein vector was created by inserting the amplified gene for NS1 into the plasmid pIND(GFP)SP1 (Invitrogen). The *sac* I cut site in pIND(GFP)SP1 is located at the extreme C-terminal end of the eGFP gene. The pIND(GFP)SP1 plasmid was digested with 10 units *sac* I (Promega). The digested plasmid was treated with 1 unit shrimp alkaline phosphatase (SAP) for 1 hour, then the SAP was heat inactivated at 65°C for 1 hour. The dephosphorylated plasmid was ligated to the digested NS1 amplicon overnight at 14°C using 1 unit T4 DNA ligase (Invitrogen), and used to transform DH5-α chemically competent *E. coli* (Invitrogen). Colonies were screened for correct orientation of the insert using PCR with primers specific for each side of the ligation site.
2. Expression of GFP/NS1 fusion protein

Either eGFP/NS1 or pIND(GFP)SP1 was cotransfected with the receptor plasmid pVGRXR into Hep G2 cells or Hep 3B cells. To accomplish transfection, 3 µg of each plasmid were diluted into 125 µl Opti-Mem 1™ (Invitrogen), along with three µl PLUS reagent™ (Invitrogen), and incubated 15 minutes at room temperature. 13 µl of Lipofectamine™ (Invitrogen) 125 µl of Opti-Mem 1 was added to the DNA and allowed to form complexes for 15 minutes at room temperature. The DNA mixture was added to cells in one well of a six-well plate containing 1 ml of Opti-Mem 1, and incubated overnight. The transfection medium was exchanged for growth medium, and expression of eGFP/NS1 or eGFP was induced by the addition of 10 µg/ml ponasterone A in 10 µl 100% ethanol. Protein expression was monitored by fluorescence microscopy.

3. Western blotting

Cells were lysed in 1% (w/v) SDS, 4M urea and 0.7M 2-mercaptoethanol. DNA was sheared by centrifugation for 1 minute at 16,000xg through a Qiashredder homogenizer column. Lysates were subjected to SDS-PAGE through 7.5-14% acrylamide gels (BioRad) at 100 V. Proteins were transferred to nitocelluose membranes for 1 hour at 100 V in a Transblot apparatus (BioRad). eGFP and eGFP/NS1 proteins were detected by immunoblotting with anti-GFP polyclonal rabbit antiserum (Invitrogen), and ECL+ chemiluminescence (Amersham). Washing and detection was performed as described in section 2.B.5.
4. Detection of apoptosis

Hep G2 or Hep 3B cells were grown on glass coverslips and transfected with pVGRXR and either eGFP/NS1 or pIND(GFP)SP1. 24 hours post-transfection, coverslips were removed from culture and covered with 2 µl annexin-V-Alexa fluor 594 (Molecular Probes, Eugene, OR) in 100 µl binding buffer (Oncogene). The coverslips were incubated for 15 minutes at room temperature in the dark, and then washed twice with binding buffer. Analysis of annexin-V binding was performed using fluorescence microscopy. Transfected cells were identified by green fluorescence and examined for apoptosis using a 528-553 nm excitation filter and a 600-660 nm barrier filter to allow for detection of red fluorescence.

5. Caspase inhibition assays

Cells were transfected and examined as for annexin-V staining. The cell-permeable caspase 3 inhibitor DEVD-FMK, caspase 5 inhibitor WEHD-FMK, or caspase 9 inhibitor LEHD-FMK (Oncogene) was added to the cell culture medium at a concentration of 500 µM 6 hours before addition of ponasterone A. The inhibitors were maintained in the culture until the cells were examined for apoptosis.

6. Immunoprecipitation and chromatin immunoprecipitation

Either eGFP/NS1 or pIND(GFP)SP1 was cotransfected with pVGRXR into Hep G2 cells, and protein expression was induced as described above. 24 hours post-induction, $10^7$ cells were lysed with 200 µl of denaturing lysis buffer consisting of 1%
SDS in TE buffer. Lysis with this buffer releases cellular DNA. The lysate was centrifuged through a Qiashredder (Qiagen) at 16,000x g for 1 minute, shearing the DNA. Lysates were then mixed with 2 ml of 1% triton-X 100 (Fisher) in PBS containing protease inhibitors (Sigma protease inhibitor cocktail). Lysates were incubated on ice for 5 minutes, and then centrifuged at 16,000xg for 15 minutes. Supernatants were removed and transferred to new tubes. 25 µl of anti-GFP polyclonal antibody (Rockland) was added to each tube, and the mixture was allowed to bind for 14 hours at 4°C in an end-over-end rotator.

After antibody binding, 60 µl of 50% slurry of Immunopure™ Protein-G agarose beads (Pierce) were added to each tube and incubated at 4°C for three hours. The tubes were centrifuged 1 minute at 4,000x g and the supernatants discarded. Immunoprecipitates were washed 5x with 1% triton-X 100 in PBS, and once with PBS alone. Immunoprecipitates were denatured by boiling for 5 minutes under reducing conditions in 1% SDS, 4M urea and 0.7 M 2-mercaptoethanol. The tubes were centrifuged at 16,000xg for one minute and the supernatants subjected to SDS-PAGE on a 7.5-14% polyacrylamide gel. The proteins were electroblotted onto nitrocellulose for 1 hour, using a Bio-Rad Ready-gel transfer apparatus at 100 volts. eGFP/NS1 was detected by immunoblotting with anti-GFP antibody (Invitrogen) as described. Antibodies specific for poly(ADP ribose) (PAR) (Pharmingen), RPA (BD biosciences), and ATR (Cell Signaling Technologies) were used at 1:5000 dilutions to detect PAR, RPA, and ATR, respectively.

For chromatin immunoprecipitation, cellular DNA of 10^7 cells was metabolically labeled with 10 µCi α^{32}P thymidine triphosphate in supplemented hepatocyte wash
medium for 18 hours. The cells were washed 3x with hepatocyte wash buffer. Radioactively-labeled cells were cotransfected with pVGRXR and either eGFP/NS1 or pIND(GFP)SP1, and protein expression was induced with ponasterone A. After 24 hours of protein expression, immunoprecipitation was performed as described. One aliquot of each immunoprecipitate was treated with 10 units DNase (Roche) for 1 hour at 37°C. After SDS-PAGE, proteins were transferred to nitrocellulose and used to expose Kodak MS film for 18 hours. The membranes were then incubated with anti-GFP polyclonal antibody. Antibody binding was detected with ECL+ chemiluminescence to visualize the location of the eGFP/NS1 fusion protein and eGFP.

7. Immunofluorescence

Hep G2 cells were grown on coverslips, transfected, and induced with ponasterone A as described above. 24 hours post-induction, growth medium was removed and cells were fixed with 4% (w/v) paraformaldehyde in PBS. Cell membranes were permeablized with 0.2% triton-X 100 in PBS for 15 minutes at room temperature, and washed 3x with PBS. Nonspecific binding was blocked by incubation of the coverslips in 10% goat serum in PBS for 1 hour at room temperature. Antibodies specific for replication protein A (RPA), PCNA (BD Biosciences), or the phosphorylated forms of p53 and CHK-1 (Cell Signaling Technologies) were diluted 1:500 in PBS and incubated with the coverslips overnight at 4°C. Nonimmune sera (Oncogene) were used as negative controls. Coverslips were washed 3x with PBS, then incubated with a 1:500 dilution of anti-rabbit IgG-Alexa fluor 594 (Molecular Probes) in PBS for 1 hour. Coverslips were
again washed 3x with PBS, and fluorescence was analyzed using epifluorescence microscopy.

**8. Treatment with pharmacologic agents**

Hep G2 cells were cotransfected with pVGRXR and either eGFP/NS1 or pIND(GFP)/SP1 and pretreated with a dilution series from 8 to 14 mM caffeine (Sigma) for 3 hours before induction of protein expression. Caffeine was maintained on the cells during expression of eGFP or eGFP/NS1. 24 hours post-induction, cells were stained with annexin-V and analyzed for apoptosis. As a control for the effectiveness of the caffeine, immunofluorescence for phospho-Chk-1 was performed using wells treated with 2 mM hydroxyurea and either 14 mM caffeine or no caffeine.

PARP was inhibited by incubating transfected cells with 5-aminoisoquinolinone (Calbiochem) at 250, 25, 2.5, and 0.25 µM concentrations 3 hours prior to induction of transgene expression. The inhibitor was maintained on the cells throughout the experiment.

**C. Results**

**1. Transfection with eGFP-NS1 construct**

To examine whether NS1 was sufficient to induce apoptosis, NS1 was cloned into a vector expressing NS1 as a fusion protein with eGFP. The vector system utilized an ecdysone-inducible promoter to allow regulated expression (Figure 3.1). This vector system allowed precise regulation of NS1 expression by the addition of ponasterone A to
Figure 3.1. Plasmid map of eGFP/NS1. The plasmid contains the gene for NS1 fused to the C-terminal end of eGFP. The promoter element consists of the heat shock minimal promoter with the SP1 enhancer elements. The plasmid is selectable in *E.coli* with ampicillin and in mammalian cells with geneticin.
Figure 3.1
the culture medium. The DNA sequence of the NS1 portion of the eGFP-NS1 plasmid was sequenced using an Amersham Megabase Capillary DNA Sequencer. Very few differences in the sequence were detected compared to the published genome sequence\textsuperscript{168}. Specifically, there were six conservative mutations, where although there was a nucleotide difference in the DNA, the amino acid coded for was identical. Two amino acid substitutions from the published sequence were found, with no insertions, deletions, or frameshift mutations (Figure 3.2).

Cells transfected with the parental vector, pIND(GFP)SP1, which express eGFP upon addition of ponasterone A to the culture medium, were used as controls for the effects of transfection and eGFP in our experiments. Transfection was achieved with approximately 2% of cells expressing the fusion protein, as determined by GFP fluorescence. Both the eGFP/NS1 and the eGFP transfected cells exhibited bright green fluorescence (Figure 3.3). The relative amounts of protein expression before and after induction with ponasterone A was examined by western blotting. Very little fusion protein was seen before induction. Expression of eGFP/NS1 increased upon addition of ponasterone A, with maximal expression detected twelve hours post-induction.

Western blotting with anti-GFP antiserum showed a specific doublet at approximately 100 kd and a larger molecular weight band at 200 kd, possibly a dimer of the NS1 fusion protein (Figure 3.4). Since these western blots were carried out under denaturing and reducing conditions, for a dimer to be present on the western blot it would
Figure 3.2 Amino acid sequence of cloned NS1. Plasmid DNA was isolated from 3 clones expressing eGFP/NS1. The DNA was sequenced in both forward and reverse reactions. The amino acid sequence was translated from sequenced DNA. Amino acids in the published sequence that differ from the cloned NS1 are indicated above the corresponding amino acid.
MELFRGVLQVSSNVLDANDNWWCLLDLDTSDLWPLHTHNLMAIYLSSVASKLDF

TGGPLAGCLYFFQVECNKFEEGYHIHVVTGGPGLNPRNLTVCEGLFNNVLYHLVTE

NVKLKFLPGMTTKGYFRDGEQFIENYLMKIPLNVVWCVTNIDGYIDTCISATFRRGA

CHAKKPRITTAINDTSSDAGESSGTGAEVVPFNGKGTKASIKFQTMVNWLCENRVFTE

F

D

DKWKLVDLNQYLSSSSHSFSQIQSAKLAIYKATNLVPTSTFLHHTDFEQVMCIKEN

KIVKLLLCQNYDPLLGQHVLLKIDDKCGKNTLWFYGPPSTGTKNLAMAIAKSVPY

GMVNWNENNFPNDVAGKSLVWDEGIKSTIVAAKAILGGQPTRVDQKMRGSVASV

PGVPVVITSNGITFVVSNGNTTVHAKALKERMVKLNFTRCSPDMGLLTEADVQQW

LTWCNAQSWDHYENWAINYTDFPGINADALHPDLQTTPITDTSISSSGGESSEE
Figure 3.3. True-color fluorescence image of transfected cells. eGFP/NS1 and PIND(GFP) transfected Hep G2 cells were visualized by fluorescence microscopy. eGFP/NS1 transfected cells (left) show punctuate nuclear staining, while fluorescence is distributed throughout the cell in the eGFP-transfected cells (right).
Figure 3.3

eGFP/NS1
eGFP
Figure 3.4. Western Blot and immunoprecipitation analysis of eGFP/NS1 fusion protein. Hep G2 cells were transfected with eGFP/NS1 or pIND(GFP) alone. Cells were lysed with SDS and either electrophoresed through a 4-15% polyacrylamide gel, or immunoprecipitated with anti-GFP antibodies, then subjected to SDS-PAGE. GFP-containing proteins were detected by western blotting with anti-GFP antiserum. **A.** Time course showing induction of eGFP/NS1 after treatment with ponasterone A. Expression is maximal at 12 hours. **B.** Immunoprecipitation of expressed proteins using anti-GFP antibodies. eGFP/NS1 is detectable as a doublet at 100 kilodaltons and an additional 200-kilodalton band. eGFP can be seen as a 37 kd band.
Figure 3.4
Figure 3.5. Cellular localization of eGFP/NS1. Hep G2 cells were transfected with eGFP/NS1, and fluorescence was visualized by fluorescence microscopy. eGFP/NS1 is predominantly located in the nucleus, with some cells fluorescing in a perinuclear or cytoplasmic manner. Cell nuclei were visualized by staining with Hoechst dye, which exhibits blue fluorescence upon binding to DNA.
necessarily be covalently linked, and not through disulfide bonds. Similarly to the
dimerization observed here, the NS1 protein from MVM dimerizes in an ATP-dependent
manner before being transported to the nucleus\textsuperscript{130}. The ATP requirement for
dimerization may be to form a covalent bond. However, the mechanisms of dimer
formation remain to be investigated.

\section*{2. Localization of NS1-GFP fusion protein}

Once transfection and protein expression had been performed successfully, the
intracellular localization of the fusion protein was examined. The eGFP/NS1 fusion
protein was predominantly located in the nucleus, with some cells also demonstrating
cytoplasmic and perinuclear staining (Figure 3.5). In pIND(GFP)SP1-transfected cells,
eGFP was found throughout the cell. Interestingly, many of the cells demonstrating
expression of NS1 showed aberrant chromatin, with condensed nuclei or seemingly
absent nuclear staining. Condensed nuclei are a hallmark of apoptosis. The aberrant
appearance of the nuclei of NS1-transfected cells could be indicative of either apoptosis
or interactions between NS1 and the DNA.

eGFP/NS1 localized to the nucleus of transfected cells in a distinct pattern of
small spots. Confocal microscope investigation of the other cellular components
comprising these spots demonstrated that NS1 colocalizes with PCNA (Figure 3.6).
PCNA is part of the DNA replication machinery, and also the DNA damage response
pathway. The association of PCNA with NS1 could indicate that there is a direct
interaction between NS1 and PCNA, or that PCNA is recruited to sites of DNA damage
induced by NS1 protein. PCNA colocalization with NS1 has been observed in MVM and
Figure 3.6. eGFP/NS1 colocalizes with the nuclear factor PCNA. Hep G2 cells were transfected with eGFP/NS1. 24 hours post-induction, cells were fixed and stained with specific antibodies to PCNA, followed by secondary antibodies conjugated to alexa-fluor 594. Fluorescence was visualized by laser confocal microscopy. Colocalization of eGFP/NS1 with both PCNA was detected. Results are representative of two different experiments, with at least three fields studied per experiment
Figure 3.6

Anti-PCNA antibody

Isotype control antibody

Untransfected PCNA antibody

eGFP/NS1  PCNA  Merge
Figure 3.7. Expression of eGFP/NS1 induces apoptosis in Hep G2 cells. Cells expressing either eGFP/NS1 or eGFP were incubated 24 hours, then stained with annexin-V conjugated to alexa-fluor 594 (red). A. Apoptotic cells stain red around the border of the cell, while eGFP/NS1 or eGFP is detectable by green fluorescence. B. Quantification of annexin staining experiments. EGFP/NS1 induces significantly more apoptosis than eGFP alone p< 0.0006. N=3, independent experiments, error bars represent standard deviation.
Figure 3.7A
Figure 3.7 B
H-1 parovirus-infected cells as part of the APAR body. Nuclear localization and colocalization of the eGFP/NS1 fusion protein with PCNA in transfected Hep G2 cells suggests that the fusion protein is functional and acting in the same manner as would be expected for wild-type NS1.

3. Induction of apoptosis

After verification that eGFP/NS1 was expressed and functioning as expected, this vector was used to investigate the role of NS1 in apoptosis of hepatocytes. eGFP/NS1 expression induced apoptosis in 35% of transfected cells, as measured by annexin-V staining (Figure 3.7), an approximately five-fold increase over cells expressing eGFP alone. The increase in apoptosis in eGFP/NS1 transfected cells compared to cells transfected with the parental vector is significant, with a student’s $t$ test $p$ value of <0.0006 after three experiments. This result demonstrates the ability of NS1 to induce apoptosis in the liver-derived cell line Hep G2. The percentage of cells undergoing apoptosis is similar to that seen in Hep G2 cells and primary hepatocytes infected with B19, suggesting that B19 and NS1 induce apoptosis in a similar manner. The use of NS1 in an expression system devoid of any other components of B19 indicates that NS1 is sufficient to induce apoptosis, providing support for the hypothesis that B19-induced apoptosis proceeds as a result of NS1 expression.

4. Caspase involvement

There are multiple apoptotic pathways, and these pathways are regulated by specific caspases. The TNF receptor family member-induced cell death pathways
activate caspase $^{16,113}$. The caspase 8-dependent apoptotic pathway differs from the endogenous pathways that induce apoptosis, for example in response to DNA damage or growth factor withdrawal. The latter are transduced through the mitochondria, and utilize caspase 9.

The experiments in chapter 2 investigating caspase involvement in B19-induced apoptosis demonstrated that B19 infection induced apoptosis in hepatocytes in a caspase 3 and 9 dependent manner. To determine if NS1 induces apoptosis through the same pathways, Hep G2 cells were transfected with either eGFP/NS1 or pIND(GFP)SP1. Transfected Hep G2 cells were incubated with specific caspase inhibitors. DEVD-FMK was used to inhibit caspase 3. Caspase 9 was inhibited with LEHD-FMK. Cultures were treated with the caspase inhibitor WEHD-FMK as a control for the involvement of nonspecific caspase inhibition.

Cultures treated with WEHD-FMK or DMSO alone as a control for the solvent that the caspase inhibitors are dissolved in demonstrated no decrease in apoptosis, while in those cells treated with DEVD-FMK or LEHD-FMK there was a significant decrease in apoptosis, almost to background levels (Figure 3.8). These findings indicate that in hepatocytes, NS1 induces apoptosis through a caspase 3 and 9 dependent pathway. The necessity of caspase 9 for the induction of apoptosis suggests that NS1 is inducing apoptosis in these cells through an intrinsic mechanism, such as DNA damage. The identification of caspase 9 as the important caspase in NS1-induced apoptosis provides evidence that NS1 activates apoptosis in the same manner as B19 infection.
Figure 3.8. **Caspases 3 and 9 are required for NS1-induced apoptosis.** Transfected cells were incubated with DEVD-FMK to inhibit caspase 3, LEHD-FMK to inhibit caspase 9, or WEHD-FMK to inhibit caspase 1, 4, and 5. Protein expression was induced with ponasterone A and apoptosis assayed by annexin staining. DEVD-FMK and LEHD-FMK significantly inhibited apoptosis (p<0.006, and p<0.008, respectively), while WEHD–FMK had no effect. N=3 independent experiments
Figure 3.8

The figure shows a bar graph comparing the percentage of apoptotic cells under different conditions. The x-axis represents caspase inhibited at various time points (DMSO, 3, 5, 9), and the y-axis represents the percentage of apoptotic cells. The graph compares two conditions: eGFP-NS1 (solid black bars) and eGFP (open white bars).
Figure 3.9. TNF-α and fas are not involved in NS1-induced apoptosis of hepatocytes. Incubation of transfected cells with anti-TNF-α or anti-fas ligand antibodies had no effect on apoptosis (n=3). The lack of inhibition was not due to lack of activity of the antibody, since the anti-TNF-α antibody was able to block apoptosis induced by the addition of recombinant TNF-α in the presence of cycloheximide.
Figure 3.9
Although caspase 9 was essential for apoptosis of both B19 infected and NS1-transfected cells, it remained possible that TNF-α or the TNF-R family member fas was also playing a role in NS1-induced apoptosis, as has been suggested for erythroid cells. To investigate the role of TNF-α and fas in NS1-induced apoptosis of liver cells, Hep G2 cells were transfected with eGFP/NS1 and treated with neutralizing antibodies against either TNF-α or fas ligand (Oncogene, BD respectively). Although the anti-TNF-α antibody was able to inhibit apoptosis induced by recombinant TNF-α, the percentage of transfected cells undergoing apoptosis was unchanged in the presence of either antibody (Figure 3.9). It is highly unlikely, therefore, that either TNF-α or fas play a significant role in NS1-induced apoptosis of liver cells.

5. Covalent attachment of NS1 to chromosomal DNA

After verifying that NS1-induced apoptosis proceeds through a caspase 9 dependent pathway, and specifically eliminating a role for TNF-α in this form of apoptosis, the involvement of DNA damage in NS1-induced apoptosis was examined. The nonstructural proteins from the parvoviruses MVM and H-1 parvovirus have been shown to have helicase and DNA binding activity, both of which have the potential to damage chromosomal DNA as well as to facilitate viral replication. MVM NS1 binds covalently to DNA as part of the replication process\textsuperscript{51,127,152}. In addition, NS1 from MVM and H-1 parvovirus colocalizes with the cellular DNA repair machinery\textsuperscript{10,47,212}. If the cellular DNA was damaged by the activities of NS1, apoptosis would be expected to ensue. A broken DNA strand with a protein attached would activate either the nick repair...
pathway or the bulky adduct pathway of DNA repair. Both of these pathways have the potential to induce apoptosis. Damage of chromosomal DNA by parvoviral proteins has not been directly demonstrated, except in the case of specific integration of AAV, although DNA nicking has been hypothesized as a possible mechanism of cell cycle arrest in MVM-infected cells.  

To discover whether or not NS1 becomes covalently linked to chromosomal DNA, cellular DNA was analyzed via chromatin immunoprecipitation. Hep G2 cells were metabolically labeled with $^{32}$P-thymidine triphosphate for 18 hours. Labeled cells were transfected with the eGFP/NS1 expression vector or the pIND(GFP)SP1 vector alone. eGFP and eGFP/NS1 were immunoprecipitated using anti-GFP antibodies, and the immunoprecipitates were washed extensively. The immunoprecipitates were dissolved in sample buffer and electrophoresed. Proteins were transferred onto nitrocellulose and analyzed by autoradiography. The location of eGFP/NS1 was determined by immunodetection with anti-GFP antiserum.  

Radioactive activity was found in specific bands that perfectly overlapped with bands formed by eGFP/NS1, but not eGFP as revealed by western blotting (Figure 3.10A). The colocalization of the radioactive signal with NS1 shows that DNA is bound to NS1 in the lysate. The denaturing methods used both in the immunoprecipitation and in the preparation of the samples for SDS-PAGE ensured that DNA could not have been present with the NS1 fusion protein unless covalently linked. Furthermore, treatment of the immunoprecipitate with DNase before SDS-PAGE decreased the specific signal by 63%, indicating that the radioactive label is DNA, and not from another source such as
Figure 3.10. DNA is covalently bound to NS1 protein. Hep G2 cells were labeled with $^{32}$P thymidine. Labeled cells were transfected with eGFP/NS1 or eGFP, and cells were lysed with SDS. Immunoprecipitation was performed on the cell lysates using anti-GFP antibodies, and the immunoprecipitate was extensively washed. Precipitates were eluted with sample buffer containing 1% SDS, electrophoresed through a 7-14% gradient polyacrylamide gel, and transferred to nitrocellulose. A. Membranes were used for autoradiography, and then probed with anti-GFP to detect eGFP-tagged proteins. The presence of a band colocalizing at 100 kilodaltons in both the autoradiograph and the western blot indicates labeling of NS1 with DNA. B. To confirm that the radioactive signal was from DNA, an aliquot of the immunoprecipitate was treated with DNase. DNase treatment abrogates the radiographic signal by 63% The figures shown are representative of 3 independent experiments.
<table>
<thead>
<tr>
<th>Autoradiograph</th>
<th>Western Blot</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFP/NS1</td>
<td>eGFP</td>
</tr>
<tr>
<td>eGFP</td>
<td>eGFP/NS1</td>
</tr>
</tbody>
</table>

Figure 3.10 A

100 kd

37 kd
Figure 3.10 B
phosphorylation of NS1 (Figure 3.10 B). These experiments provide direct evidence that NS1 nicks and covalently binds to cellular DNA. This type of DNA damage would be expected to initiate both the bulky adduct DNA damage response pathway and the single strand nick response pathway.

6. DNA damage response

DNA damage response proteins block the cell cycle in response to DNA damage and are capable of inducing apoptosis if the DNA lesion is not repaired. It is a logical hypothesis, therefore, that NS1 induces apoptosis through damaging cellular DNA. There are multiple types of DNA damage repair pathways. The bulky adduct repair pathway involves the kinase ATR, which autophosphorylates upon binding DNA damage-sensing complexes. ATR phosphorylates proteins that initiate a cascade leading to cell cycle arrest, DNA repair, and apoptosis. In addition to the adduct repair pathway, the pathway responsible for the repair of DNA nicks is potentially involved in NS1-induced apoptosis. This pathway is mediated through poly(ADP ribose)polymerase (PARP). Upon binding DNA nicks, PARP transfers poly(ADP ribose) (PAR) chains to many of the surrounding proteins, leading to DNA repair and a decrease in the ATP levels of the cell. If the damage to the DNA is extensive, both the adduct repair and nick repair pathways may result in apoptosis.

a. Bulky adduct repair pathway

The involvement of the ATR-mediated DNA repair pathway in NS1-induced apoptosis was examined in eGFP/NS1-transected cells by inhibiting ATR with caffeine.
The concentrations of caffeine used effectively inhibited the kinase activities of ATR, as shown by a decrease in phosphorylation of the ATR substrate CHK-1 following caffeine treatment (Figure 3.11B). Incubation of eGFP/NS1-transfected cells with caffeine inhibited apoptosis in a dose-dependent manner, reducing the percentage of apoptotic cells by 70% at a concentration of 14 mM (Figure 3.11). The decrease in apoptosis upon treatment with caffeine confirms that NS1 damages DNA, and identifies DNA damage as the major determinant of NS1-induced apoptosis.

To confirm the involvement of ATR in NS1-induced apoptosis, the interactions between NS1 and RPA were studied. RPA is a DNA-binding protein that serves as the recognition factor for bulky adduct damage. RPA binds with high affinity to single stranded or damaged DNA, and bound RPA recruits other repair molecules\(^\text{142,143}\). One result of RPA binding to DNA is activation of the ATR-dependent DNA repair process\(^\text{208}\). Immunofluorescence staining for RPA in eGFP/NS1-transfected Hep G2 cells demonstrated remarkable colocalization of RPA with eGFP/NS1, indicating that NS1 is found in areas of the cell that contain DNA damage capable of activating ATR (Figure 3.12). The micrograph shown is representative of three independent experiments. RPA colocalized with NS1 in all cases where NS1 was found in the nucleus.

The finding that NS1 damages DNA in a manner which would generate bulky adducts suggests that RPA would bind to the DNA damage induced by NS1. RPA probably does not bind directly to NS1, since immunoprecipitated eGFP/NS1 complexes do not contain RPA (data not shown), but RPA binding to DNA that was made single stranded either through the distortion of DNA structure caused by the covalent binding of NS1 or by the unwinding action of the helicase activity of NS1 would be sufficient to
Figure 3.11. The DNA adduct repair pathway is necessary for NS1-induced apoptosis. A. Hep G2 cells transfected with eGFP/NS1 were treated with caffeine at concentrations from 8 to 14 mM. Caffeine inhibits the ATR-dependent DNA repair pathway. Apoptosis was assayed using annexin-V staining. The decrease in apoptosis compared to cells without caffeine treatment was significant at a concentration of 10 mM, with a p value < 0.009. The data were derived from 3 independent transfections.

B. Caffeine inhibits ATR. Hep G2 cells were treated with 2 mM hydroxyurea and either incubated or not with 14 mM caffeine. 24 hours after the addition of hydroxyurea, cells were fixed and antibodies specific for the phosphorylated state of the ATR substrate CHK-1 were used in immunofluorescence staining. Cells with phosphorylated CHK-1 have bright red nuclei.
Figure 3.11 A
Figure 3.11 B
Figure 3.12. eGFP/NS1 colocalizes with RPA. Hep G2 cells were transfected with eGFP/NS1. 24 hours post-transfection, cells were fixed and incubated with anti-RPA antibodies. Secondary antibodies conjugated to alexa-fluor 594 were used to stain RPA. Laser confocal micrograph showed colocalization of eGFP/NS1 (green) with the DNA damage sensing protein RPA (red). No signal was seen when an isotype control antibody was used in place of the anti-RPA antibody, or when the primary antibody was omitted.
Figure 3.12
initiate ATR activation. The colocalization of RPA with NS1 provides further evidence implicating the ATR-dependent repair pathway in eGFP/NS1-transfected cells, supporting the data generated by incubation of transfected cells with caffeine.

Activated ATR phosphorylates the kinase CHK-1, which in turn activates a multitude of proteins, among them p53. p53 is an important cell cycle regulatory molecule, serving to halt the cell cycle in cells with damaged DNA. Activated CHK-1 phosphorylates p53, stabilizing and activating the transcription-enhancing effects of p53. Possible consequences of the activation of p53 include cell cycle blockade or apoptosis.

To determine the necessity for p53 in NS1-induced apoptosis, transfected Hep G2 cells were examined for the presence of phosphorylated p53 by immunofluorescence. There was no increase in phosphorylation of p53 in response to eGFP/NS1 expression as compared to eGFP-transfected cells, although there was an increase in phospho-p53 in response to the DNA damaging agents hydroxyurea and zeocin. To further investigate the role of p53 in NS1-induced apoptosis, the cell line Hep 3B was transfected with either eGFP/NS1 or pIND(GFP) alone. Hep 3B is a liver carcinoma cell line that does not express p53, while Hep G2 cells express p53 normally\textsuperscript{149}. Expression of eGFP/NS1 in Hep 3B cells induced apoptosis at a similar proportion to expression in Hep G2 cells, with 41% of cells undergoing apoptosis in response to NS1 transfection (Figure 3.13). The ability of NS1 to kill cells that do not express p53 demonstrates that although ATR activity is important for NS1-induced apoptosis, p53 is dispensable. A Potential mechanism of ATR-induced apoptosis that does not involve p53 includes the activation of p63 or p73 by ATR.
Figure 3.13. p53 is not necessary for B19-induced apoptosis.  Hep 3B cells, which do not express p53, were transfected with eGFP/NS1 or pIND(GFP), and assayed for apoptosis with annexin-V staining 24 hours after induction of transcription.  Significantly more eGFP/NS1 transfected cells were apoptotic than pIND(GFP) transfected ($p<0.002$).
Figure 3.12

The bar graph shows the percentage of apoptotic cells for two conditions: eGFP-NS1 and eGFP. The graph indicates a significant increase in apoptotic cells for the eGFP-NS1 condition compared to eGFP.
b. Nick repair pathway

Although the ATR-dependent DNA repair pathway is essential for NS1-induced apoptosis, other DNA damage repair pathways exist that can also effect apoptosis. One such pathway is the single-strand nick repair pathway, initiated by PARP. NS1 is known to nick viral DNA, potentially implicating the nick repair pathway in NS1-induced apoptosis. Recognition of single strand nicks is achieved by PARP, which upon binding to broken DNA ends becomes activated and poly(ADP)ribosylates multiple surrounding proteins. Activation of PARP has been demonstrated to induce apoptosis in neuronal cells, to interfere with the electron potential of the mitochondria, and to be required for the translocation of AIF from the mitochondria to the nucleus\textsuperscript{200,213}.

To study the involvement of the PARP-initiated DNA repair pathway in NS1-induced apoptosis, the cell-permeable PARP inhibitor 5-aminoisoquinolinone was added to eGFP/NS1-transfected Hep G2 cells. Inhibition of PARP significantly reduced apoptosis in eGFP/NS1-transfected cells in a dose-dependent manner when compared to eGFP/NS1-transfected cells treated with DMSO alone. Inhibition of apoptosis was maximal at 57% at a concentration of 25 µM (Figure 3.14). This finding demonstrates that PARP activation, and therefore the PARP-induced DNA repair pathway, is involved in NS1-induced apoptosis. The involvement of PARP provides additional data that DNA damage is responsible for NS1-induced apoptosis.

As a method of confirming the involvement of PARP activation in NS1-induced apoptosis, immunoprecipitation was performed on lysates from eGFP-NS1 transfected cells. After electrophoresis and blotting, the membranes were probed with an anti-PAR antibody. PAR is the polymer that activated PARP.
Figure 3.14. **PARP activity is necessary for optimal NS1-induced apoptosis.** Hep G2 cells were transfected with eGFP/NS1, or pIND(GFP) and gene expression was induced for 24 hours in the presence or absence of the PARP inhibitor 5-aminooisoquinolinone. Addition of the inhibitor reduced apoptosis in a dose-dependent fashion, ($p<0.003$), as assayed by annexin-V staining. N=3. Addition of 5-aminooisoquinolinone had no effect on the eGFP transfected cells.
Figure 3.14

5-aminoisoquinolinone concentration (micromolar) vs. apoptosis %

- eGFP/NS1
- eGFP

Legend:
**Figure 3.15. NS1 is Poly(ADP ribose)ylated.** Hep G2 cells were transfected with eGFP/NS1 or pIND(GFP)SP1. Immunoprecipitation was performed on cell lysates using anti-GFP antibodies, and the proteins from the immunoprecipitates were probed with anti-PAR antibodies. eGFP-NS1 protein was ribosylated, as shown by a band at 100 kd in the eGFP-NS1 transfected, PAR probed sample. eGFP is not ribosylated, as evidenced by the lack of a band at 37 kd in the eGFP only lane. Blots shown are representative of three independent experiments.
Figure 3.15
transfers to neighboring proteins in response to DNA damage. eGFP/NS1 was poly(ADPribose)ylated, while eGFP was not (Figure 3.15). The only way for NS1 to become poly(ADPribose)ylated is to have been in contact with active PARP. The finding that NS1 is poly(ADPribose)ylated directly demonstrates not only that PARP interacts with NS1, but also that PARP is active in eGFP/NS1 transfected cells. The association of NS1 with activated PARP further supports the data generated with the PARP inhibition studies indicting PARP as an important factor in NS1-induced apoptosis.

D. Conclusion

The experiments presented in this chapter demonstrate that an inducible, GFP-linked NS1 expression system is a valuable tool for studying the interactions of NS1 with the host cell. The eGFP/NS1 vector allows experiments to be performed that examine single transfected cells, experiments utilizing bulk cultures in which the effects of NS1 can be masked by nontransfected cells. The inducible nature of the system allows sufficient survival time to perform experiments on the transfected cells.

The eGFP/NS1 fusion protein localizes in the cell in the same manner as the wild-type protein would be expected to do. Upon expression, NS1 traffics to the nucleus, where it associates with the DNA replication factors PCNA and RPA. The association of these factors with NS1 is similar to the association patterns found in MVM and H-1 parvovirus APAR bodies.

Over one third of eGFP/NS1-transfected cells undergo apoptosis, as demonstrated by annexin-V staining. Furthermore, the mechanism of NS1-induced apoptosis is similar to that observed in B19-induced apoptosis. Both B19 infection and NS1 transfection
induced apoptosis in approximately one third of cells, and both infection and transfection are dependent on caspases 3 and 9 to effect cell death. The necessity for caspase 9, along with the known DNA-interacting activities of NS1, make DNA damage a likely mechanism for B19 and NS1-induced apoptosis.

Damage to DNA can result in DNA repair, leading to continued viability of the cell, or failure to appropriately repair the DNA, leading to apoptosis. Colocalization of NS1 with the DNA repair enzymes RPA and PCNA suggest that DNA repair is occurring at the sites of NS1 localization. That NS1 actually damages DNA by covalent binding is clearly demonstrated by the chromatin immunoprecipitation assays showing colocalization of NS1 with radiolabeled DNA. This finding is further supported by the data which show that RPA colocalizes with NS1 and that NS1 is poly(ADPribose)ylated. These experiments demonstrate the activation of the ATR and PARP-mediated DNA repair pathways, respectively.

The importance of DNA damage to NS1-induced apoptosis is shown by the abrogation of apoptosis in the presence of inhibitors of these DNA damage recognition enzymes. Both the single strand nick repair pathway, mediated by PARP, and the bulky adduct pathway, mediated by ATR, are necessary for optimal induction of apoptosis induced by NS1 expression, since inhibition of these pathways significantly decreases apoptosis in transfected cells. Although it is possible that NS1 uses mechanisms other than DNA damage to induce apoptosis, the degree of inhibition seen when these inhibitors are utilized suggests that DNA damage is the primary mechanism for NS1-induced apoptosis. Furthermore, the most likely pathway NS1 may use to induce
apoptosis in a non DNA-damage dependent manner, TNF-α, was not involved in NS1-induced apoptosis of transfected hep G2 cells.

The chromosomal DNA-damaging activity of NS1 has interesting implications for B19 infection. Since DNA damage has the potential to lead to apoptosis in multiple cell types, it is likely that B19 infection of other tissues may also generate DNA lesions, leading in part to the variety of diseases associated with B19 infection.
Chapter 4

Discussion and Future Directions
The experiments presented in this dissertation provided data that support the following conclusions:

1. B19 infects Hep G2 cells and produces RNA for NS1 but not VP1 or VP2.
2. B19 infects primary hepatocytes and produces RNA for NS1 but not VP1 or VP2.
3. NS1 transcripts are translated, with NS1 protein localizing to the nuclei of infected cells.
4. B19 infection causes apoptosis in both primary hepatocytes and Hep G2 cells.
5. Irradiated B19 is unable to induce apoptosis in hepatocytes, suggesting that transcription is required for cytotoxicity.
6. B19-induced apoptosis of hepatocytes is dependent on caspases 3 and 9 but not caspase 8.
8. The eGFP/NS1 vector system is valuable in investigating the effects of NS1 on a single-cell level.
9. Expressed eGFP/NS1 protein localizes in the cell nucleus, and exists as three polypeptides of differing molecular weights.
10. Expression of NS1 causes apoptosis in liver-derived Hep G2 cells, without the presence of any other viral components.
11. NS1-induced apoptosis is dependent on caspases 3 and 9.
12. NS1 covalently binds to DNA.
13. NS1 is poly(ADPribose)ylated, indicating activation of PARP by NS1-induced DNA damage.

14. PARP activation is required for optimal NS1-induced apoptosis.

15. NS1 colocalizes with the ATR-activating protein RPA.

16. The ATR-dependent DNA repair pathway is involved in NS-1 induced apoptosis.

These findings have a variety of implications for B19-induced disease and the molecular virology of B19 infection. The data derived from the infection and apoptosis experiments answer major questions concerning the capacity of B19 to cause AFLF. The demonstration of NS1-induced DNA damage and the involvement of DNA repair in NS1-induced apoptosis has implications for clinical treatment of AFLF, and suggests mechanisms through which B19 could induce autoantibodies or autoimmune disease.

A. Parvovirus B19 causality of acute fulminant liver failure

B19 has been associated with hepatitis since its discovery in a pool of serum samples being tested for hepatitis B\textsuperscript{39}. Numerous reports and studies have implicated B19 in pathological processes of the liver, including post-transplantation liver dysfunction\textsuperscript{92,169}, hepatitis\textsuperscript{69,102,140,145,194,211}, and fulminant liver failure\textsuperscript{84,91,117,178,196}. Although multiple reports have associated B19 with liver disease, two papers disputed the association of B19 with AFLF. Eis-Hübinger and colleagues detected B19 DNA in explanted livers from 17 of 43 (40\%) individuals undergoing orthotopic liver transplantation for chronic liver disease of defined etiologies\textsuperscript{57}. Although they did not include patients with AFLF in their study for comparison, they concluded that the high
prevalence of B19 in patients with diseases not caused by B19 signifies that B19 is not involved in liver disease.

Wong and colleagues used nested PCR to detect B19 genomes and reported no difference in the prevalence of liver B19 DNA in fulminant hepatitis (35%) versus hepatitis B or C virus infections (33%). However, their data show a significant difference when fulminant hepatitis (35%) was compared to biliary atresia (5%)\(^{205}\). In their hands, liver samples from individuals with hepatitis-associated anemia had a low prevalence (9%) of B19 DNA. These results suggested to them that B19 was not implicated in AFLF or hepatitis. The experiments conducted by Eis-Hübinger et al. and Wong et al. are not directly comparable to those conducted by Karetnyi and Langnas for a variety of reasons, including the age of the patients, the definition of AFLF utilized as a study criterion, and the controls utilized by each research group (for a full comparison, see section 1.H.3).

Eis-Hübinger’s and Wong’s findings that B19 may persist in liver parallels the findings of B19 persistence in synovium\(^{77,177}\), skin\(^{100}\), liver, and in bone marrow from adults with chronic B19 arthritis\(^{62,176}\). The significance of the presence of latent B19 DNA in these tissues, however, is not well understood. B19 DNA persistence may occur without causing deleterious effects to the cell, as demonstrated by these reports showing viral DNA with no sign of any disease.

B19 likely remains latent for a period of time before reactivation or a second hepatic insult leads to clinical liver disease. There are a number of findings that support reactivation of B19 as the cause of B19-induced liver disease. Failure to detect anti-B19 IgM antibody in patients with AFLF and B19 DNA may represent reactivation of latent
infection\textsuperscript{91}, rather than a new infection. A new infection would be expected to be marked by high specific IgM titers. Similarly, in one report, B19 DNA but not capsid protein was detected in myocardium from an infant with fulminant myocarditis\textsuperscript{138}. The absence of capsid protein suggests that the infection was not ongoing, but rather that the DNA may have been latent from a previous infection.

Although the presence of B19 DNA in livers from patients without AFLF can be explained by viral persistence, the finding of parvovirus B19 DNA in healthy livers or diseased livers where the cause of the disease is known not to be B19 complicates the requirements to prove causation of AFLF by B19. Finding B19 DNA in livers from healthy individuals does not, however, eliminate the virus as a candidate causative agent for liver disease.

Proof of causation of illnesses by microorganisms has historically been predicated on fulfilling Koch’s postulates. As presented by Fredricks and Relman\textsuperscript{63}, Koch’s postulates are:

1. The parasite occurs in every case of the disease in question and under circumstances which can account for the pathological changes and clinical course of the disease.
2. The parasite occurs in no other disease as a fortuitous and nonpathogenic parasite.
3. After being fully isolated from the body and repeatedly grown in pure culture, the parasite can induce the disease anew.

Although these postulates are effective in establishing criteria for proving causation of many bacterial diseases, viruses, especially viruses capable of latency and differential
clinical presentation, present problems if Koch’s postulates are to be enforced as the only standard to establish causation.

In particular, Koch’s postulates are difficult to apply to B19 infection. B19 DNA is not found in every case of AFLF, complicating the first postulate. B19 is also capable of persisting for years in a variety of tissues. The ability to persist without causing disease decreases the utility of the second postulate. B19 DNA may be found in nondiseased tissues, but is certainly pathogenic, as is also the case with many other latent viruses, such as EBV and herpes simplex virus. The inability of viruses to replicate outside of a cell precludes their isolation in pure culture, an important component of the third postulate. Further complicating the third postulate as applied to B19 disease is the fact that B19 will only infect humans, making replication of disease after isolation in pure culture impossible, or at least very unethical.

Despite these difficulties, Koch’s postulates can serve as guidelines for a context to evaluate B19 causation of AFLF. Evans\textsuperscript{59} proposed a more statistical approach to the evaluation of viral causation, where a statistical increase in disease following exposure to the putative pathogen as compared to unexposed controls is substituted for always finding the pathogen. The studies by Langnas\textsuperscript{91} et al. and Karetnyi\textsuperscript{84} et al. fulfill this condition by demonstrating a significant increase in B19 DNA in liver samples from AFLF patients as compared to control patients.

The second postulate, finding the putative pathogen only in the diseased tissues, does not necessarily apply to B19-induced disease, given the persistent nature of B19 infection. However, Karetnyi et al. found active viral infection in AFLF patients, as demonstrated by B19 RNA, but not in control patients\textsuperscript{84}. If active infection is substituted
for the presence of DNA, the finding of RNA fulfills Koch’s second criterion for establishing causation.

The experiments presented in this dissertation come as close as possible to evaluating B19 infection of liver with respect to the third postulate as possible with the currently available reagents. Although B19 cannot be grown in pure culture, the control conditions used in the infections with B19, such as the NCL-PARVO-treated inoculate and the UV-irradiated inoculate fulfill the same purpose. The finding that there is no increase in apoptosis upon inoculation with these control sera demonstrates that the effects of infection on the Hep G2 cells is due to B19 and not another factor, fulfilling the same purpose as passaging the virus in pure culture.

Although there is no animal model for B19 infection, and therefore no way to replicate the disease process with isolated viruses, the *in vitro* experiments discussed in this dissertation demonstrate the pathologic potential of B19 infection of the liver. B19 has never before been shown to have a cytopathic effect on cells that are nonpermissive for productive replication. The demonstration that B19 infection induces apoptosis in hepatocytes suggests a mechanism through which B19 could damage multiple tissue types, leading not only to fulminant liver failure, but also to the variety of diseases associated with this virus.

The experiments utilizing inoculation of hepatocytes with B19 described in this dissertation go beyond merely finding B19 DNA in clinical samples to demonstrate that B19 is able to infect liver cells with subsequent production of NS1 protein and induction of apoptosis. These findings establish that B19 is not simply a passenger in liver cells,
but has the capacity to cause cell death, replicating the disease state in a model, albeit an
*in vitro* system.

RT-PCR and immunofluorescence studies demonstrated that B19 is able to infect
hepatocytes. The experiments analyzing infected cells demonstrate clearly that the
presence of B19 infection or NS1 expression is sufficient to cause apoptosis in
hepatocytes, even without production of new B19 particles.

AFLF is marked by apoptosis of hepatocytes, so the pathogenic processes seen *in
vitro* in these experiments correlate very well with what is seen *in vivo* in B19-associated
AFLF. In the absence of an *in vivo* model, the demonstration of the ability of B19 to
infect and kill hepatocytes is as close to proving hepatotoxicity of B19 infection and
fulfilling the third of Koch’s postulates as possible *in vitro*. Although the formal proof of
B19 causation of AFLF remains elusive, the considerable clinical evidence linking B19
infection with hepatitis is greatly strengthened by the mechanistic studies presented
herein. In the presence of both clinical and mechanistic data implicating B19 in AFLF,
and the demonstrated fulfillment of the criteria for establishing causation, B19 should be
considered capable of causing AFLF, and evaluated seriously for its ability to cause the
variety of other diseases associated with AFLF.

**B. NS1 expression and apoptosis**

NS1 is central to the ability of B19 to induce apoptosis. Analysis of B19
infection utilizing RT-PCR and immunofluorescence demonstrated that in hepatocytes,
B19 produces NS1 protein, but not VP1 or VP2. Irradiated B19, which contains the viral
DNA and capsid proteins but which is unable to transcribe the NS1 gene, is unable to induce apoptosis.

The association of B19-induced apoptosis with NS1 expression was further strengthened by experiments using the eGFP/NS1 expression vector. This vector solved a number of problems usually associated with studies of all parvoviral nonstructural proteins, and NS1 of B19 in particular. The inducible nature of the NS1 expression vector allowed for tight control over NS1 expression, enabling studies to be performed independently of the often toxic effects of transfection reagents. The inducing agent, ponasterone A, is nontoxic, unlike the agents used in other attempts to generate inducible NS1 expression vectors in the past, which used heavy metal or human steroid inducible vectors. The lack of cytotoxic effects of the inducer on mammalian cells is extremely important given that the readout of the experiments utilizing the vector is cell death. The labeling of NS1 with eGFP allowed studies to be carried out on a single-cell level, overcoming the difficulties associated with transfection of liver cells. This vector system will continue to be valuable in the study of NS1.

eGFP/NS1 expression induced apoptosis in transfected cells, and did so in a similar proportion of cells as underwent apoptosis in response to B19 infection. The finding that both infection and transfection induce apoptosis in approximately the same percentage of cells suggests that both transfection with NS1 and infection with B19 induce apoptosis through the same mechanism. Augmenting the correlation between NS1 expression and B19 infection in apoptosis induction is the finding that both systems utilize the same caspases to cause cell death. These findings support the biological
relevance of the NS1 expression vector as a tool to analyze the mechanisms of B19-induced apoptosis.

Implication of NS1 as the causal agent of B19-induced apoptosis allows the investigation of the molecular pathogenesis of B19 infection to be focused on the probable activities of NS1. NS1 expression has two major effects in infected cells, transcriptional activation and modification of DNA structure. Transcriptional activation would be expected to lead to apoptosis through the production of TNF-α, while DNA damage would lead to apoptosis through DNA damage response pathways.

The particular activity of NS1 responsible for cell death was first analyzed by studying the effects of caspase inhibition on NS1-induced apoptosis. Apoptosis in both B19-infected and NS1-transfected cells proceeds through a caspase 3-and 9-dependent pathway. Caspase 8 activity is not necessary for B19-induced apoptosis, suggesting that the action of B19 on Hep G2 cells is a result of an apoptotic pathway initiated within the infected cell rather than as a result of exogenous signal of a TNF receptor family member. Indeed, neutralization of TNF-α activity or fas ligand has no effect on NS1-induced apoptosis. This result differs from published findings that B19 sensitizes erythroid cells to apoptosis through TNF-α receptor binding. Although these results do not directly argue against the findings of that report in that B19 infected liver cells may be more sensitive to TNF-α, TNF-α is not necessary in B19-induced apoptosis of hepatocytes.

Also of considerable interest when considering the mechanisms of B19-induced apoptosis is the finding that hepatocyte growth factor decreases apoptosis in primary hepatocytes infected with B19. Hepatocyte growth factor is known to increase the
concentrations of anti-apoptotic molecules such as Bcl-X<sub>L</sub>.<sup>60</sup> These apoptotic regulators function through controlling mitochondrial membrane potential, which controls caspase 9-dependent apoptosis. The fact that hepatocyte growth factor is able to abrogate apoptosis of infected cells suggests a link between infection and mitochondrial apoptosis. Hepatocyte growth factor also induces more efficient DNA repair. Increased efficiency of DNA repair would decrease the apoptosis initiated by B19 infection only if the initiating signal were DNA damage. In combination, the results of the caspase inhibition studies, the TNF-α neutralization experiments, and the effects of adding hepatocyte growth factor to infected cells strongly suggest that NS1 induces apoptosis in B19 infected hepatocytes through damaging the DNA of the cell.

**C. B19 infection and DNA damage**

The enzymatic activities of NS1 that could lead to DNA damage include endonuclease, helicase, and nucleotide triphosphate binding activities<sup>107,109</sup>. Since many of these activities involve modification of viral DNA, it is logical to hypothesize that NS1 may also modify cellular DNA. NS1 from MVM nicks and then covalently attaches to the DNA strand. If nicking of DNA and covalent attachment of NS1 to cellular DNA were to occur, these processes would generate two types of lesions, single strand nicks and bulky adducts. Two different pathways carry out repair of these types of DNA lesions. The nick repair pathway utilizes PARP as the damage recognition molecule. PARP is capable of binding to single strand nicks and initiating repair. If the damage is extensive, PARP can initiate apoptosis by altering the mitochondrial membrane<sup>121,175</sup>.
The bulky adduct repair pathway is dependent on the kinase ATR. Upon activation, ATR phosphorylates a variety of substrates, including CHK-1, p53, and p73, each of which further transduces signals that result in DNA repair or apoptosis\textsuperscript{171,174}. The data presented in this thesis implicate both the nick repair and bulky adduct repair pathways in the induction of B19-induced apoptosis.

Demonstration of DNA damage by NS1 is of paramount importance. The presence of NS1-induced DNA damage suggests that parvovirus-induced interference with the cell cycle could be a product of the cellular DNA damage response. Visualization of NS1-induced DNA damage would also support the hypothesis that the apoptosis seen upon B19 infection or expression of NS1 is due to DNA damage. The chromatin immunoprecipitation experiments presented in this dissertation demonstrate conclusively that NS1 covalently binds to cellular DNA. Covalent attachment of NS1 to DNA would generate both a nick in the DNA strand and a bulky addition to the DNA. Both of these types of DNA lesions can lead to apoptosis under appropriate conditions.

The single strand nick repair pathway is implicated in NS1 through the activity of the protein that initiates single strand nick repair, PARP. Treatment of NS1-transfected cells with the PARP inhibitor 5-aminoisoquinolinone decreases apoptosis. Furthermore, NS1 is directly poly(ADP ribose)ylated, indicating the presence of activated PARP in the immediate vicinity of NS1. PARP, therefore, is directly responsible for a subset of NS1-induced apoptosis, implicating the single strand nicking capability of NS1. PARP-induced apoptosis proceeds through disruption of the mitochondria, allowing the release of caspase 9 and apoptosis-inducing factor. Studies using caspase inhibitors implicated caspase 9 in both B19 and NS1-induced apoptosis. The mechanisms of PARP-induced
apoptosis are consistent with the involvement of caspase 9 that is seen in B19-induced apoptosis.

Single strand nicks are the type of DNA damage that would be expected from a parvovirus nonstructural protein as it separates the DNA replicative form into individual genomes. The process of nicking by NS1 in MVM is highly regulated, depending on both cellular factors, such as RPA, and recognition by NS1 of a specific DNA sequence\textsuperscript{98}. The involvement of single strand nicks in B19 NS1-induced apoptosis of hepatocytes suggests that either nicking of DNA by the NS1 protein of B19 is not as highly regulated as NS1 from MVM, and so is able to proceed on cellular DNA, or that there are sites suitable for NS1 nicking on the chromosomes. Since the NS1 DNA binding site is only 4-6 base pairs long\textsuperscript{66}, finding the NS1 binding sequence at several sites in the genome should not be surprising. Further work analyzing the site at which NS1 binds to the chromatin should demonstrate the NS1 binding sequence.

Although PARP is important in B19-induced apoptosis, inhibition of the ATR pathway in NS1-transfected cells leads to a greater decrease in apoptosis than inhibition of PARP. The involvement of the bulky adduct repair pathway in NS1-induced apoptosis further confirms the direct demonstration of covalent DNA binding by NS1. The ability to inhibit apoptosis through the inhibition of ATR demonstrates that B19 utilizes the normal DNA damage repair pathways to induce apoptosis, and not a unique virus-specific interaction. The eGFP/NS1 expression vector will be useful in determining the site on NS1 that is necessary to covalently bind to DNA.

The ability of B19 to kill cells by damaging their DNA answers many questions about B19 pathogenesis. The relatively small number of cells killed by NS1 expression or
**Figure 4.1. Model for NS1-induced apoptosis.** B19 virus binds to globoside on the cell surface. NS1 DNA traffics to the nucleus, where transcription of the NS1 gene occurs. NS1 protein is translated, and locates to the nucleus. NS1 protein nicks and covalently attaches to the DNA. The helicase activity of NS1 unwinds the DNA, leading to single-stranded regions. RPA binds to the single-stranded DNA, and PARP binds to the NS1-induced single-strand nicks. RPA activates ATR, leading to apoptosis through the mitochondrial pathway. PARP becomes active, causing a drop in cellular ATP and NAD, and induces apoptosis through the mitochondrial pathway.
Figure 4.1
B19 infection can be explained by the DNA damage-apoptosis paradigm, since those cells that die are most likely those that are unable to adequately repair their DNA. If the NS1 was directly acting on apoptosis-inducing factors, a higher death toll would be expected. The finding that NS1 induces apoptosis through damaging DNA also explains how the presence of B19 could be either deleterious or innocuous to the cell. In cells where NS1 is not expressed, perhaps because of the lack of cellular factors that regulate transcription, B19 DNA would be innocuous. However, if transcription were resumed, the expression of NS1 could attack the host DNA, with cytotoxic effects on the cells in which NS1 becomes activated.

In addition to effects on the cell, NS1 protein covalently binding to chromatin has implications for the viral life cycle. The mechanisms regulating parvoviral DNA packaging are not known. NS1 attachment to cellular DNA suggests that viral NS1 would also covalently bind to viral DNA, and allows for the possibility that NS1 is the molecule that regulates packaging of viral DNA into the capsid. Almost nothing is known about B19 assembly, so the role of NS1 in packaging must for now remain speculation, but the covalent association of NS1 to DNA presents support for an NS1-mediated model of DNA packaging.

D. Clinical Implications

The data in the preceding chapters provide strong evidence supporting the assertion that B19 is one of the causal agents of fulminant liver failure. B19 should be considered in cases of fulminant liver failure with associated aplastic anemia. The linkage between B19 infection and AFLF has potential therapeutic value, since most
commercial preparations of human immunoglobulin contain neutralizing anti-B19 antibodies. Treatment of patients with AFLF with this anti-B19 serum could attenuate liver damage during B19 infection. Although immunoglobulin therapy has been utilized to treat chronic B19 infection in HIV patients, it has never been used to attempt to attenuate liver damage or other pathogenic effects of B19 infection. Immunoglobulin therapy, however, would require active, productive infection with B19 in order to be useful. If the reactivation model of B19-induced liver damage is accurate, this type of therapy would not be expected to make a difference.

This work, however, also shows direct evidence of DNA damage by NS1, and that that damage leads to cellular apoptosis. DNA-damage induced pathology has been successfully treated using PARP inhibitors in cases of ischemic damage. Since PARP activation is involved in B19-induced apoptosis, it is possible that PARP inhibitors could have a therapeutic effect on patients undergoing B19-induced liver damage, even in the absence of viremia.

The other DNA repair pathway initiated by B19, the ATR-dependent pathway, is also interesting in a therapeutic setting, although not as a target for inhibition. NS1-induced apoptosis is not dependent on p53. There has been interest in using parvoviruses as anticancer agents given their oncosuppresive tendencies. Many anticancer therapeutic agents rely on p53 to transduce the cytotoxic signal. Multiple cancer cell lines, however, are null for p53 expression. If the data seen here concerning the mechanisms of cell death induction apply to other parvoviruses, it increases the potential utility of parvoviruses as antitumor agents because they do not rely on p53 to kill cells.
The covalent bond between NS1 and DNA suggests a mechanism for the generation of autoantibodies in B19 infection. B19 infection often results in the production of anti-DNA autoantibodies\textsuperscript{72,99}. Although the role of these antibodies in disease pathogenesis is not known, B19 may mimic systemic lupus erythematosus, which is marked by anti-DNA antibody production.

The origin of anti-DNA antibodies has always been a difficult question for immunologists, since T cells will generally only become stimulated by peptides associated with the MHC complex, and T-cell stimulation is often necessary for the activation of B-cells and antibody production. However, a viral protein with a covalently linked DNA strand could serve as a dual antigen, with autoreactive B cells recognizing the DNA portion, internalizing the complex, and presenting peptides derived from NS1 to virus-specific T cells. NS1-specific T cells, generated in the normal immune response to B19, could then activate autoreactive B-cells that presented NS1 on their MHC molecules, leading to the development of autoantibodies (Figure 4.2).

This model for the generation of autoantibodies in response to B19 infection is attractive in that it assumes the utilization of T cells that are specific for viral NS1 protein. The problem of the generation of autoreactive T cells is thereby avoided. T cells specific for NS1 which are activated in the immune response against B19 would be sufficient to activate B cells to initiate the production of autoantibodies. Clinically, it is interesting when considering this model that patients with high titers of anti-NS1 antibodies also are more prevalent to develop arthropathy, a condition which may be a result of autoantibody production\textsuperscript{81,104,215}. 
Figure 4.2. Model for generation of anti-DNA antibodies in B19 infection.  

A. NS1-DNA complexes are bound by DNA specific autoreactive B-cells.  
B. The NS1-DNA complex is internalized and the NS1 is proteolytically degraded. Degraded NS1 peptides bind to MHC class II molecules and are transported to the cell surface.  
C. NS1-specific CD4+ T-cells recognize NS1 peptides in the context of MHC class II molecules.  
D. The T-cell activates the B-cell through ligation of CD40 and cytokine production. The activated B cell generates anti-DNA autoantibodies.
A. DNA-specific B cell binds DNA-NS1 complex

B. NS1 is processed and presented on MHC

C. NS1-specific T-cell binds to NS1 peptide on B cell MHC

D. T-cell activates B cell to produce anti-DNA antibodies

Figure 4.2
Another potential connection between B19 infection, DNA damage, and autoimmunity can be found in the case of rheumatoid arthritis. A hallmark of rheumatoid arthritis is the generation of point mutations in the genome of the pannus, or fibroblast-like infiltrating cells, of the affected joints\textsuperscript{93,156}. B19 infection has been associated with rheumatoid arthritis\textsuperscript{189}. DNA from B19 persists in synovial tissue\textsuperscript{77,123,215}, and inoculation with B19 has been shown to increase the invasiveness of synovial fibroblasts\textsuperscript{154}. It is possible that B19 influences synovial fibroblasts through DNA damage. DNA damage is inherently mutagenic. Although only 1/3 of cells that express NS1 undergo apoptosis, it is likely that the cells that do not undergo apoptosis also sustain DNA damage, possibly in a mutagenic fashion. Mutation of cellular DNA by NS1-induced DNA damage has a variety of potential effects on the cell. There is currently no data on whether increased mutation rates are seen in B19-infected cells, or what effect mutation has on these cells. The role of NS1 mutagenesis is an area which requires further study.

Many of the diseases associated with B19 infection are autoimmune in nature. NS1-DNA interactions have the potential to explain the development of autoimmunity after B19 infection, but the linkage remains to be demonstrated. The interactions between B19 infection and autoimmunity therefore require further study.

**E. Conclusion**

The goal of the research presented here has been to investigate the cytotoxic potential of B19 in liver cells, and, if cytotoxicity was found, the mechanisms through which cell death proceeds. The research succeeded by demonstrating that infection of hepatocytes by B19 does in fact lead to cellular apoptosis. Further, the pathway from
infection to cell death was shown to be through NS1-induced DNA damage, followed by caspase 9-dependent apoptosis. The work has interesting implications for multiple areas of B19 virology and causation of B19-associated diseases, and provides tools and information that can be utilized to investigate B19 infection and pathogenesis in still greater depth.
Literature Cited


34. Christensen, J., S. F. Cotmore, and P. Tattersall. 2001. Minute virus of mice initiator protein NS1 and a host KDWK family transcription factor must form a
precise ternary complex with origin DNA for nicking to occur. J. Virol. 75:7009-7017.


51. **Dettwiler, S., J. Rommelaere, and J. P. Nuesch.** 1999. DNA unwinding functions of minute virus of mice NS1 protein are modulated specifically by the lambda isoform of protein kinase C. J. Virol. 73:7410-7420.


113. Muzio, M., A. M. Chinnaiyan, F. C. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J. D. Bretz, M. Zhang, R. Gentz, M. Mann, P. H. Krammer,
M. E. Peter, and V. M. Dixit. 1996. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. Cell 85:817-827.


EDUCATION
PhD in Life Sciences with an emphasis in immunology
Pennsylvania State University
Expected Graduation August 2004

B.S. in Microbiology
Brigham Young University
August 1999

HONORS AND ACTIVITIES
Intercollege Graduate Student Outreach Award, Pennsylvania State University, 2004
Arthritis Foundation trainee award and grant 2002, 2003
Life Science Consortium Fellow, Pennsylvania State University, 1999-2001
Trustees Scholar, Brigham Young University 1993-1999

PUBLICATIONS
Poole, Brian; Grote, Amy; Zhou, Jing; and Naides, Stanley. Nonstructural Protein of Parvovirus B19-induced Apoptosis of Liver-Derived Cells. Manuscript in preparation.
Poole, Brian and Naides, Stanley. DNA damage and Repair in Parvovirus B19-Induced apoptosis. Manuscript in preparation.

ABSTRACTS PUBLISHED
Poole, Brian and Naides, Stanley. Parvovirus B19 Infection Induces Apoptosis in Nonpermissive Cells. FASEB Journal 16:4 abstract 238.22
Poole Brian, Karetnyi Youry, and Naides Stanley. Parvovirus B19 Induction of Caspase 3-Dependent Apoptosis in Liver-Derived Cells. IX international parvovirus Workshop, Bologna Italy, 2002 (Oral Presentation)
Poole, Brian D. and Stanley J. Naides Directed Cell Killing by Viral Proteins Inducing Apoptosis. Innoventure 2003, Hershey PA
Poole, Brian and Naides, Stanley. The nonstructural protein of B19 induces apoptosis in nonpermissive cells through a caspase 3 and 9 dependent pathway. American College of Rheumatology Annual scientific meeting. Orlando, FL October 24-28, 2003