INFECTIOUS CAPABILITY OF THE PLUS (+) AND MINUS (-) STRANDS OF PARVOVIRUS LUIII

By

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ABSTRACT

The replication of autonomous parvovirus LuIII and its encapsidation pattern have not been fully explained. Two genomic clones of LuIII, pBLu(+) and pBLu(-), were constructed. These clones were used to produce the (+) or (-) ss viral DNA, to assess the capability of infection of the individual strands. Three different techniques were utilized to obtain the single-stranded LuIII DNA. Only the treatment with *Exonuclease III* yielded both strands from the pBLu(+) construct. Each polarity of the single-stranded LuIII DNA was independently transfected into cancerous HeLa cells by electroporation. The results obtained suggest that both the ss(+) and ss(-) LuIII DNA were capable of establishing an infection in these cells. The (-) strand produced 100% cell lysis two days prior to the (+) strand, appearing to be more effective in the establishment of viral infection.

RESUMEN

La replicación y el patrón de encapsidación del parvovirus LuIII no han sido explicados completamente. Dos clones genómicos de LuIII, pBLu(+) y pBLu(-), fueron construidos. Estos clones fueron utilizados para tratar de producir ADN viral de hebra sencilla de polaridad (+) o (-). Se intentaron tres técnicas diferentes para obtener el ADN viral de hebra sencilla de LuIII. Solamente el tratamiento con *Exonucleasa III* produjo ambas hebras utilizando la construcción pBLu(+). Ambas polaridades del ADN de hebra sencilla de LuIII fueron transfectadas a células cancerosas HeLa por electroporación. Los resultados obtenidos sugieren que las hebras de ADN de polaridad (+) y (-) de LuIII fueron capaces de establecer una infección en estas células. La hebra (-) alcanzó 100% de lisis celular dos días antes que la hebra (+), aparentando ser más efectiva en el establecimiento de la infección viral.

DEDICATION

To my marvelous family: José Antonio, Sandra, Abali, José María and Bebo…I love you endlessly.

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CHAPTER I
INTRODUCTION

Parvoviruses are small, naked, icosahedral viruses, characterized by having linear single-stranded DNA genomes with hairpin termini of approximately 5 kilobases in length. They can lytically infect cells of a wide range of animal species, including humans. Interest in these agents arises mostly from the striking specificity of the virus-host interactions.

The low genetic complexity of parvoviruses forces them to tightly depend on exogenous factors in order to complete their lytic cycle. Parvoviruses are unable to stimulate cell proliferation and consequently depend on the proliferation state of the cells. These requirements, which reflect interactions between highly regulated cellular functions and viral determinants, are likely to account for the spectrum of cytopathogenicity of parvoviruses both *in vivo* and in culture (Mousset et al., 1994; Op De Beeck et al., 1995; Op De Beeck and Caillet-Fauquet, 1997).

The need for efficient transfer of potentially therapeutic genes to defined cell populations has stimulated the development of vectors based on viruses. To date scientists have concentrated on RNA-containing Retroviruses, to address these needs. However, these viruses possess a number of disadvantages that have led to the development of vectors based on DNA-containing viruses such as Adenovirus, Herpes Simplex Virus and Parvovirus (Ali et al., 1995).

Parvoviruses are well suited for gene transfer due to their relatively small genome, and apathogenic nature. It is becoming increasingly clear that the Parvovirus-based vectors are a useful alternative to the more commonly used Retroviral vectors in human gene therapy (Podsakoff et al.,1995; Voyles, 1993; Srivastava, 1994), because they can replicate in the nucleus of rapidly dividing cells such as transformed and tumor-derived cells. Many studies have been

conducted using Adeno-associated virus (AAV) for long term gene therapy, because AAV can integrate into a specific chromosomal site, proving to have anti-tumor and anti-proliferative properties in tissue culture and in animal studies (Di Pasquale and Stacey, 1998; Hermonat, 1994; Rolling and Samulski, 1995).

Very promising results have been obtained in studies using autonomous parvovirus, more specifically the rodent parvoviruses Minute Virus of Mice (MVM) and H-1, as vectors for gene therapy, thanks to the oncotropic properties and the specificity of virus-host interactions of autonomous parvovirus. Early after their discovery in the 1960's, the Autonomous Parvoviruses of rodents were found to be associated with a marked decrease in cancer incidence in their natural or experimental host. Parvovirus infections were found to inhibit tumorigenesis in immunocompetent laboratory animals. Parvoviral oncosupression concerns both spontaneous tumors and tumors experimentally induced by oncogenic viruses, chemical carcinogens or implanted tumor cells. Parvoviruses have been suggested as a preventive anti-cancer surveillance and acts analogously to a "vaccine" against tumors (Faisst et al., 1998).

The parvovirus LuIII shares over 80% of its DNA sequence with the rodent parvoviruses MVM and H-1 (Astell et al., 1979; Astell et al., 1983), therefore it seems that humans are not their natural host, even though it was isolated from a human lung cell line (Siegl, 1976). The complete DNA sequence of parvovirus LuIII was determined and cloned in the vector pUC19 (Diffoot et al., 1993) to generate a highly infectious genomic clone, pGLu883. The availability of this clone together with the oncosupressive properties attributed to parvoviruses, make LuIII a very good subject for gene therapy studies.

In order to use a virus as a vector in gene therapy, a complete characterization of its genomic material, proteins and replication mechanisms are essential to the understanding of how these components interact in cancerous

human cells. The main objective of this research was to assess the potential of independent infection of the plus (+) and minus (-) strands, of the Parvovirus LuIII genome.

CHAPTER II
LITERATURE REVIEW

Parvoviruses are small single-stranded DNA viruses with a genome size that ranges from 4600 to 6000 nt in length. They have a non-enveloped icosahedral capsid with a diameter of 18-26 nm. These viruses, noted for their species specificity, can infect a wide range of organisms causing severe diseases. Their linear genomes contain small imperfect terminal palindromes that fold back on themselves to form complex hairpin telomeres. Parvoviruses contain two large Open Reading Frames (ORFs), one code for two non-structural proteins, NS-1 and NS-2, and the other encodes coat proteins VP-1 to VP-3. A number of morphological and physicochemical properties characterize the family Parvoviridae, along with distinct features of genome organization and replication (Berns, 1996; Corsini et al., 2001; Lukashov and Goudsmit, 2001).

Classification

Currently the classification of parvoviruses is primarily based on their host range and whether or not they depend on helper functions of other viruses to replicate, accordingly parvoviruses are separated into three groups: autonomous parvoviruses of vertebrates, helper-dependent viruses of vertebrates, and autonomous viruses of insects (Faisst and Rommelaere, 2000).

The family is taxonomically organized into the Densovirinae and Parvovirinae subfamilies. Viruses that belong to the Densovirinae infect arthropods, whereas the Parvovirinae infect vertebrates. The Parvovirinae are further subdivided into three genera, Erythrovirus, Dependovirus and Parvovirus (Lukashov and Goudsmit, 2001).

The Densovirinae subfamily is composed of viruses that replicate autonomously and infect invertebrates, several economically or medically important insect groups, such as Lepidoptera, Diptera, Orthoptera, Dyctioptera and Odonata. These viruses are known as Densonucleoviruses (DNVs), and are highly pathogenic to their hosts, causing lethal diseases in natural or laboratory insect populations. The diseases, Densonucleoses, are characterized by the accumulation of viral particles in the nuclear compartment. This subfamily includes three genera: Densovirus (*Junonia coenia* densovirus), Iteravirus (*Bombyx mori* densovirus) and Brevidensovirus (*Aedes aegypti* densovirus) (Bando et al., 1990; Bergoin and Tijssen, 1998; Dumas et al., 1992; Giraud et al., 1992; Faisst and Rommelaere, 2000).

The Parvovirinae subfamily is widely distributed among warm-blooded animals and comprises three genera: Dependovirus, Erythrovirus and Parvovirus. Even though all members of the three genera are similar in structure, they can be separated by differences in host range, pathogenesis, clinical symptoms, gene expression and replication (Berns, 1996; Faisst and Rommelaere, 2000).

The genus Dependovirus includes several viruses isolated in association with adenoviruses, hence the name Adeno-associated virus or AAV. This genera includes several human serotypes (AAV 1-5), and serotypes isolated from primates, cattle, dogs, and birds. Unlike other human viruses, AAV is naturally defective, except under special conditions, and require coinfection with a helper virus (e.g. adenovirus, herpes simplex virus, papilloma virus) to establish a productive infection. AAV appears to be non-pathogenic in humans. It is capable of infecting dividing and non-dividing cells *in vitro* and *in vivo,* as well as cells originating from different species and tissue types *in vitro* (Vincent et al., 1997).

In the absence of a helper virus, infection of AAV can result in integration of the complete viral genome into a specific site in the long arm of human chromosome 19, entering a latent state until reactivation by superinfection of the host cell with a helper virus or by genotoxic stress. This subsequent reactivation results in the rescue and replication of the viral genome, completing the life cycle of the virus. The integration of AAV into the host genome appears to have no negative effect on cell growth or morphology *in vitro* and, epidemiologically, it has been shown that seropositivity to AAV can be a protective factor against the development of uterine cervix carcinoma (Di Pasquale and Stacey, 1998; Yang et al., 1997).

The genus Erythrovirus is relatively new, and it includes human parvovirus B19 plus three distinct primate viruses, SPV (simian parvovirus from cynomolgus monkeys), PTMV (pig tailed macaque virus), and RMV (rhesus macaque virus), of which the best characterized is SPV. Members of the Erythrovirus replicate autonomously in erythroid progenitor cells, causing a transient depression in erythrocytes. Parvovirus B19 is the smallest DNA-containing virus known to infect humans (Faisst and Rommelaere, 2000).

Of all parvoviruses, B19 is the only one known to be a native human pathogen (Woolf et al., 1989). It was accidentally discovered in 1975 by Cossart et.al., and subsequent studies have determined that it binds to globoside (P-antigen of the P blood group system) on the surface of erythroid progenitor cells. B19 is the causing agent of erythema infectiosum or fifth disease; up to 80% of adults in the population are seropositive to B19. In humans with geneticly-based hemolytic diseases (e.g. sickle-cell anemia, thalassemia, and hereditary spherocytosis) that result in reduced red cell half-life, infection by B19 can cause a life-threatening aplastic crisis. Immune compromised individuals are at risk of developing a persistent B19 infection (Heegard and Hornsleth, 1995; Lukashov and Goudsmit, 2001).

The genus Parvovirus is comprised of small DNA viruses without envelopes that replicate in the nucleus of the host cell via linear, double-stranded, episomal DNA molecules, utilizing a hairpin transfer mechanism to replicate the ends of their linear DNA molecule. They are capable of replicating independently and infect a wide range of mammals such as rodents (RV, H-1, MVM), canines (CPV) and felines (FPV), among others. Due to their low genomic complexity, the parvovirus life cycle depends on cellular factors that are expressed as a function of cell proliferation and differentiation (Avalosse et al., 1996; López-Guerrero et al., 1997; Tullis et al., 1994).

MVM and H-1 are the best characterized parvoviruses, and both rodent parvoviruses have been completely sequenced and studied. They are very similar in sequence but different hairpin structures (a characteristic of all the parvoviruses) at the termini of the virion, which are known as the 5' and 3'-terminals. Both viruses encapsidate 99% minus (-) strand, and this encapsidation pattern is believed to be controlled by information on the palindromic termini. Because of the sequence homology with MVM and H-1, LuIII is believed to be a rodent parvovirus. LuIII also has different hairpin structures at the end of its virion but, interestingly, this parvovirus resembles AAV in that it encapsidates 50% of plus (+) and minus (-) strand. Contrary to the other two rodent parvoviruses, the replication model for LuIII has not been defined, and its natural host is still unknown (Berns, 1996).

Viral Particle

The viral particle of parvoviruses is composed of three proteins and a linear single-stranded DNA molecule. The protein capsid has icosahedral symmetry and a diameter of 18-26 nm. The particle has a MW of 5.5-6.2 x 10^6 daltons, 80% of the mass is protein and 20% is DNA. The buoyant density of the

intact virion in CsCl is 1.39 to 1.42 g/cm^3, which allows the separation of AAV from helper adenovirus in coinfections. The virions are very resistant to inactivation; they can survive alcohol and ether treatment, temperatures of up to 56°C for 60 min, and pH changes between 3 and 9. They can be inactivated by formaline, β-propriolactone, hydroxylamine, and oxidizing agents (Berns, 1996; Faisst and Rommelaere, 2000).

Genomic Organization

The genome of parvoviruses is a linear single polydeoxynucleotide strand with a MW of 1.55-1.97 x 10^6 daltons. The linear strand can be of either plus or minus polarity. The complete sequence of many of the parvovirus genomes has been reported over the past years. The relatedness of these genomes ranges from >90% to <30%. Among the many parvoviruses sequenced to date, it has been shown that H-1 and MVM consist of 5,176 and 5,084 bases, respectively; AAV2 contains 4,680, and the LuIII genome is 5,135 bases long. However, the overall organization of each genome includes palindromic sequences at their 5' and 3' termini of the virion strand that form secondary structures. The termini can assume T, Y or U-shapes (Figure 1) (Berns, 1996; Diffoot et al., 1989). The palindromic sequence at the 3' end of most murine autonomous parvoviruses is approximately 115 bases long, and can form a secondary structure with T- or Y-shape. In B19, this sequence has been found to be more than 300 nucleotides in length. The 5' palindromic sequence can assume a U- or T- shape conformation and can be 206-242 nucleotides in length, an exception being the 5'-end of the human B19 genome, which appears to present a terminal repeat of the sequence at the 3'-end. For some parvoviruses, the 5' terminus can exist in two possible orientations flip or flop, which are formed when several unpaired nucleotides exist within the hairpin (Figure 1). These conformations have been associated

with the efficiency of the DNA replication (Berns, 1996; Chen et al., 1988; Tattersall and Cotmore, 1988).

The remainder of the genome consists of two large open reading frames (ORFs) encoding the nonstructural and structural genes (Figure 2) (Diffoot et al., 1992). The first ORF covers much of the left half of the genome (approximately m.u. 6 to 42) and encodes for the nonstructural proteins (Rep proteins in AAV), this ORF is the first to be transcribed. Any mutation within this ORF blocks viral replication and gene expression. The second ORF occupies much of the right half of the genome (m.u. 45 to 90), and encodes the structural (coat) proteins. Up to three coat proteins have been identified in the virion, with the exception of Aleutian disease virus (ADV), which contains only two coat proteins. Mutants altered in either of the ORFs can be complemented in *trans*, however, the palindromic sequences at both termini are required in *cis* for replication to occur (Astell et al., 1983; Berns, 1996; Pintel et al., 1983).

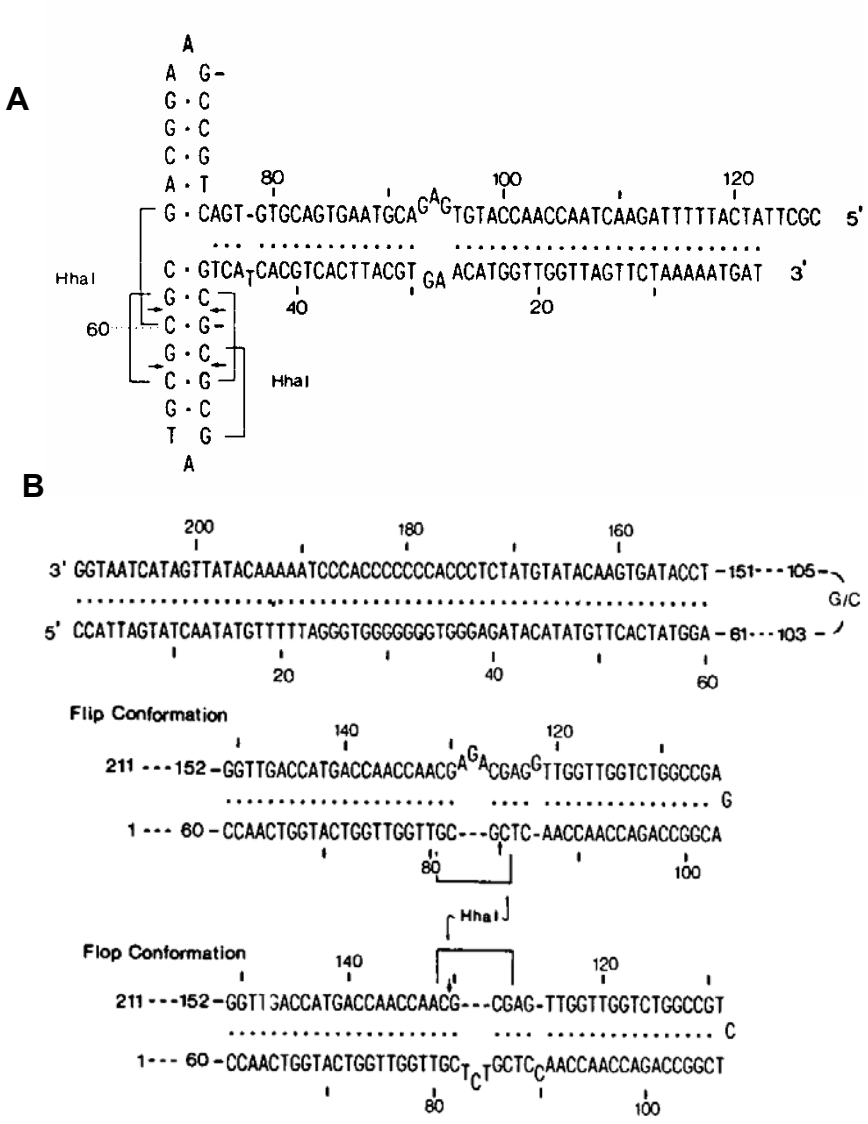


Figure 1. **Palindromic termini of Parvovirus LuIII.** (**A**) DNA sequence of the left terminus (3' end) of the minus strand of LuIII forming its characteristic T-shape secondary structure. (**B**) DNA sequence of the right terminus (5' end) of the plus strand of LuIII forming the U-shape secondary structure with its particular flip and flop conformation (Reprinted from Diffoot et al., 1989).

Transcription

The major portion of the parvovirus genomes (excluding the hairpin termini) contains two large open reading frames (ORFs). These ORFs are, by definition, on the plus strand and transcription proceeds in a left to right direction (Figure 2). Using this orientation, the left half of the DNA encodes the NS or Rep (non-structural) proteins while the right half encodes VPs (structural proteins). The pattern of gene expression varies depending on the number of promoters (1-3 promoters).

Human parvovirus B19 has two promoter like elements, of which only the one at m.u. 6 proved to be functionally active in both permissive and nonpermissive cells. The transcription of B19 initiates at P6, which is transactivated by the nonstructural protein NS1, and proceeds to the opposite end of the DNA. Complex alternate splicing and use of two polyadenylation sites (one in the middle of the genome and one at the right hand end) are responsible for the production of at least nine transcripts that encode NS and VP proteins as well as novel small proteins, the 7.5 and 11 kDa proteins. Seven of these transcripts are used as mRNAs. Of interest is that all nine B19 transcripts contain a common 56 nt leader sequence. Although the B19 transcriptional pattern seemed unusual when it was first discovered, the transcription map for bovine parvovirus is very similar (Gareus et al., 1998; Pallier et al., 1997).

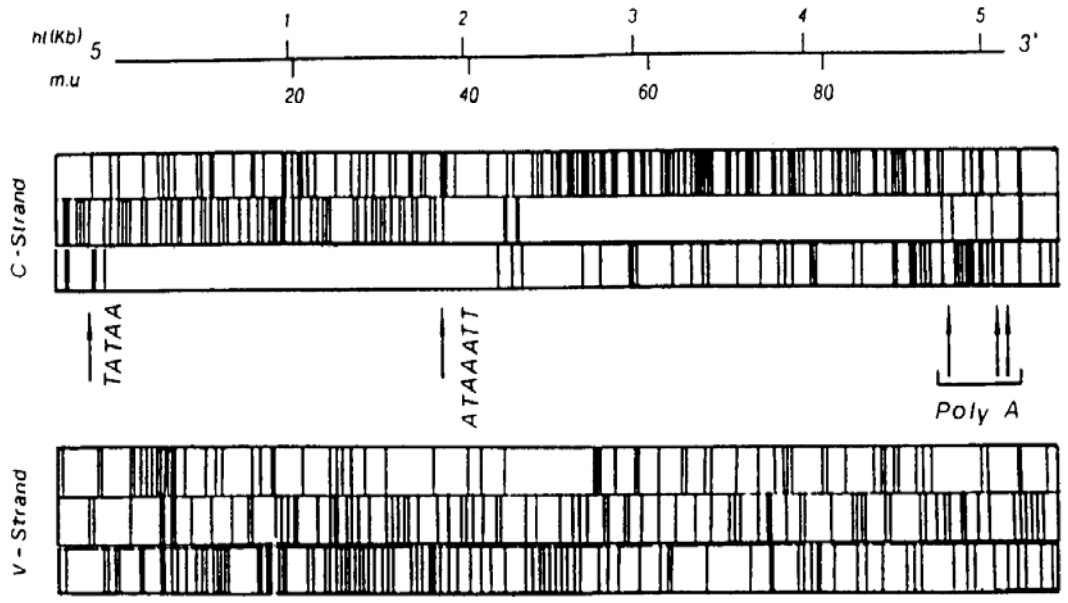


Figure 2. **Genomic organization of Parvovirus LuIII.** Stop codons of the plus (C-strand) and minus (V-strand) strands of LuIII in the three open reading frames (ORFs) are indicated by vertical lines while the positions of the promoter like sequences and polyadenilation signals are indicated by arrows. (Reprinted from Diffoot et al., 1992)

The defective parvovirus AAV makes use of three promoters, P5, P19 and P40. The replication (*rep*) gene encodes at least four proteins, Rep78, Rep68, Rep52, and Rep40 that are essential for parvoviral replication. The Rep proteins are translated from differentially spliced mRNAs that originate from transcription promoters P5 and P19. The P40 promoter generates mRNAs encoding three structural proteins: VP1, VP2 and VP3. One or more of the *rep* gene products trans-activates transcription from all three AAV promoters, for AAV2 it has been shown that the Rep proteins stimulate transcription of the P40 promoter, however, the response of P5 and P19 promoters to Rep varies depending on conditions within the host cell. In the absence of a helper virus, the Rep protein normally downregulates p5 and p19 (Berns, 1996; Renuka et al, 1995).

MVM is an example of a virus that uses two promoters. Its genome is organized into two overlapping transcription units that produce three major classes of RNA. Transcripts R1 (4.8 Kb) and R2 (3.3 Kb) are generated from a promoter (P4) at m.u. 4 and encode the viral nonstructural proteins NS1 and NS2, respectively, whereas the R3 (3.0 Kb) are generated from a promoter (P38) at m.u. 38 and encode the viral capsid proteins VP1 and VP2 (Figure 3). The third capsid protein (VP3) is not a primary translation product but is derived by proteolytic cleavage which removes the amino-terminal region of VP2 and which occurs only after capsid assembly and packaging of the viral genome. All transcripts terminate at the right end of the genome and alternate splicing controls the relative steady state level of the transcription products during the different stages of infection (Cotmore and Tattersall, 1986; Gersappe et al, 1999).

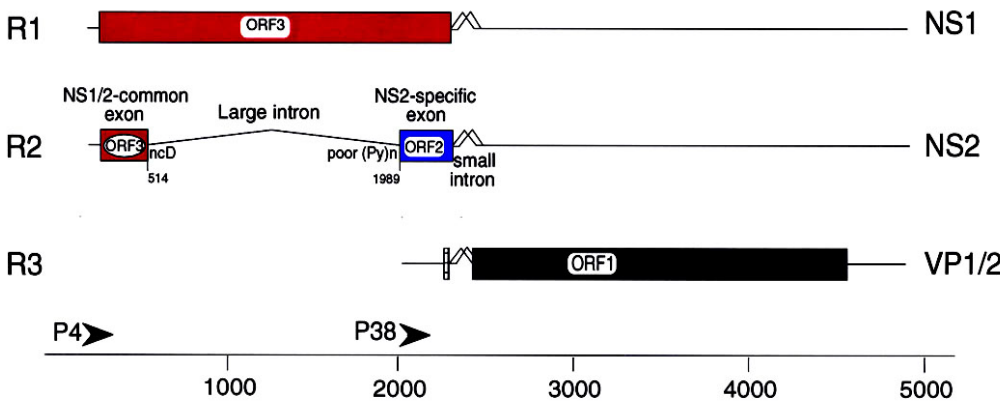


Figure 3. **Genetic map of MVM.** R1 is showing the major transcript class that encodes for the non-structural protein NS1. In R2, two open reading frames (ORF2) are used to encode for the non-structural protein NS2. R3 indicates the ORF used to encode for the two structural proteins, VP1 and VP2. The location of the two promoters P4 and P38 is shown by arrows (Reprinted from http://www.missouri.edu/~mmiwww/djp.html).

The processing of transcripts for all parvoviruses is complex. The selection of intron donor and acceptor sites results in a variable amount of transcripts. For example in MVM and AAV, the use of alternate splice acceptor sites results in synthesis of low levels of the largest coat protein (VP1) and high levels of the smaller, major coat proteins, VP2 and VP3, respectively (Berns, 1996).

Regulation of gene expression for multiple promoter viruses is controlled by *cis*-acting sequences and transacting factors (both viral and cellular). The major nonstructural protein of MVM, NS1, has been reported to have a modest stimulatory effect in P4 and has proven to be a potent activator of P38 transcription. NS1 binds to $(ACCA)_n$ motifs within the MVM genome in a DNA sequence-specific manner. The NS1 transactivation of the P38 promoter is dependent only on the presence of TATA and GC box sequences in the promoter. P38 also contains a transactivation response region (TAR) sensitive to NS1. The P4 promoter contains a functional GC box as a major element in its promoter motif, and the transcription factor Sp1 is known to bind to the GC boxes present in both the P4 and P38 promoters. Many studies suggest that the transcriptional regulation by the NS1 protein may involve an interaction with Sp1 bound to the promoter region. It has also been shown, that these proteins can interact on the absence of the promoter sequence (Gersappe et al., 1999; Krady and Ward, 1995; Lorson et al., 1996).

The temporal regulation of early and late gene expression has been studied in MVM and BPV. There is evidence that NS encoding transcripts are expressed earlier then VP encoding transcripts. However, there is not a clear demarcation between early versus late transcripts, as seen with some DNA viruses (Astell, 1999).

Non-Structural Proteins

Autonomous parvoviruses produce two non-structural proteins: NS1 (71-83 kDa) and NS2 (24-28 kDa). The protein expression occurs at a very early stage of infection, the non-structural proteins play an important role in the viral life cycle. The major non-structural protein (NS1) is a multifunctional phosphoprotein that exhibits site-specific DNA binding, nickase, helicase and ATPase activities. These activities account for the primary role played by NS1 in viral replication, which makes this protein essential for the establishment and maintenance of the replication fork. It has been suggested that, in order to coordinate these various functions, the protein is regulated by post-translational modifications, particularly phosphorilation (Nüesch et al., 2003). The sequence for the NS1 protein is contained within the virus left hand ORF, frameshift mutations in this region are lethal and prevent excision and/or subsequent replication of the viral DNA sequences. NS1 holds an activating region that induces the promoters with which it interacts and represses transcription of unrecognized promoters by a squelching mechanism. It binds to P38 specifically at TAR (P38 minimum transactivation region) in a strictly ATP-dependent manner (Berns, 1996; Christensen et al., 1995; Cotmore and Tattersall, 1988).

The small non-structural protein NS2 consist of three isoforms (NS2-P, -L, and –Y) that differ at their carboxy termini as a result of alternative splicing events. This protein exists in phosphorylated and unphosphorylated forms that are mainly located in the cytoplasm of infected cells; however nonphosphorylated NS2 can also be found in the nucleous. The role of NS2 is still elusive; it has been previously reported to play a critical role in viral capsid assembly and consequently in the generation of viral single-stranded DNA, and in viral mRNA translation. Functioning in coordination with NS1, it is an enhancer of cytotoxicity in human cells (Cotmore et al., 1997; Eichwald et al., 2002). Experiments have shown the capacity of NS1 to kill murine and human neoplastic cells by virtue of

cytotoxic activity of this protein, which results from its accumulation inside the cell. Others have documented that parvoviral regulatory proteins confer different inhibitory actions upon the host cell, these include: suppression of *in vitro* transformation, inhibition of tumor growth in derived cells, and killing of some transformed cells by means of apoptosis (Fernandez et al., 1995; Mousset et al., 1994; Op de Beeck and Caillet-Fauquet, 1997).

Parvovirus B19 encodes only one non-structural protein, which has been detected in association with both nuclear and cytoplasmic arrays of capsids and remains associated with the complete viral particle until its release from the cell (Gareus et al., 1998).

AAV has four replication regulatory proteins (analogous to non-structural proteins of autonomous parvoviruses): Rep 40, Rep 52, Rep 68 and Rep 78. Rep proteins are required for efficient AAV-2 replication and production of viral progeny, and are likely to be involved in the targeted integration of the viral DNA into a specific site in the human chromosome 19 (Fernandez et al., 1995; Op de Beeck et al., 1995).

Structural Proteins

The capsid of autonomous parvoviruses and Adeno-Associated Virus is formed by three major structural proteins (VP): VP1 (80-86 kDa), VP2 (64-75 kDa), and VP3 (60-62 kDa). These capsid proteins self-assemble to form mature capsids naturally or when expressed in mammalian cell lines. There are about nine VP1 subunits per particle, with the remainder of the subunits consisting of VP2 in empty (lacking DNA) capsids or a mixture of VP2 and VP3 in full virions. Particles of the parvovirus MVM contain a total of 60 individual polypeptide chains. The dominant capsid protein, VP2, is the C-terminal 64 kDa of the 83

kDa VP1 polypepetide. In infectious virions, many VP2 proteins undergo post-assembly cleavage, during entry into the host cell, removing about 20 residues from their amino termini to generate VP3. VP3 is the major structural protein, making up to 80% of the virion mass (Agbandje et al., 1998).

Not all parvoviruses encode three proteins. For Aleutian disease virus and B19, VP1 and VP2 are the solely capsid proteins. For B19, sixty copies of the structural proteins assemble into the viral capsid, the major structural protein VP2 accounts for 95% of all proteins made and VP1 is the minor structural protein. Bovine parvovirus (BPV), lapine parvovirus and some densoviruses have four structural proteins (Berns, 1996).

The capsid proteins determine the cell tropism for parvovirus infection. For example, MVMp infects fibroblasts, whereas MVMi infects T lymphocytes. This tissue tropism has been shown to be primarily determined by two amino acids in the sequence shared by the capsid proteins, VP1 and VP2. The mechanism underlying the species specificity of canine parvovirus (CPV) and feline parvovirus (FPV) appears to be similar to that of MVM. CPV can productively infect canine and feline cell lines whereas feline parvovirus (FPV) is restricted to the latter. In other studies the putative regulatory role of the parvoviral capsid has been assayed, although no detectable *trans*-regulatory effect on transcription promoters was evident (Spegelaere et al., 1994; Spitzer et al., 1997; Weichert et al., 1998).

Replication

Parvovirus DNA replication and assembly occurs in the nucleus and is coupled with the S-phase of the cell cycle. Unlike some other DNA viruses, parvoviruses are unable to induce quiescent cells to initiate DNA synthesis;

hence, parvoviral DNA replication is delayed until host cells enter on their own in a round of genomic DNA replication. Besides cellular replication factors, the parvoviral regulatory protein NS1 (Rep 68 for AAV) is essential for the replication of parvoviral DNA. Transcription of the NS gene has been reported to be S-phase dependent and may thus contribute to the coupling of viral DNA amplification with cellular DNA synthesis (Deleu et al., 1998).

In autonomous parvoviruses, the expression of NS1 is thought to require a prior replicative event, which resembles rolling circle replication (RCR), starting with the synthesis of a complementary strand primed at the genomic left-hand (3' terminal) hairpin, converting the ss virion DNA into double-stranded monomer replicative form (mRF) DNA, which can serve as transcription template. Complementary strand synthesis stops when reaching the folded-back right-hand (5' terminal) hairpin, and is followed by ligation of the newly synthesized and parental strands. This results in a molecule covalently closed at both ends (cRF). Such closed mRF molecules can only be detected after cells have entered S-phase, suggesting that conversion may be the primary S-phase associated event of the parvoviral life cycle (Baldauf et al., 1997; Bashir et al., 2000; Willwand et al., 1998) (Figure 4).

This so-called conversion reaction relies only on cellular factors present in the S-phase of the cell cycle. Subsequent amplification of RF DNA proceeds through the formation of multimeric intermediates by a unidirectional leading strand synthesis mechanism, and requires the non-structural protein NS1, to serve as site-specific duplex DNA binding protein with site-specific, single-strand nickase activity. It activates replication by binding to its duplex recognition sequence, $(ACCA)_n$, and introducing a single-stranded nick close to the core recognition site. This reaction leaves NS1 covalently attached to the 5'-end at the nick site via a phosphotyrosine bond and generates a 3' hydroxyl group pf the nucleotide at the cleavage site, which serves as primer for DNA synthesis. The

palindromic sequences play a key role in replication, because they contain the viral origins, which are not only essential for replication initiation but also necessary for resolution of replicative intermediates, breaking them down to unit length genome duplexes (Christensen and Tattersall, 2002; Nüesch et al., 1998).

The left-end palindrome of the MVM genome can be folded to form a Y-shaped hairpin structure containing a mismatch in the stem, designated the "bubble" sequence, where a GA dinucleotide opposes a GAA trinucleotide. In addition, this palindrome contains enhancer elements for the initiating viral promoter and provides a terminal base pair with a free 3' hydroxyl group, termed *ori*L_H (for hairpin origin), which primes conversion of the genome into a monomeric duplex by cellular DNA polymerase(s)(Figure 5-A). In such monomeric duplex intermediates, however, the turnaround form of the left-end palindrome cannot function as an active origin, due to the presence of the bubble mismatch. After being replicated to the dimer intermediate, the extended hairpin forms a palindromic double-stranded sequence termed the dimer junction, because it bridges two unit length head-to-head duplex genomes (Figure 5-B). In the dimer junction, the nucleotides of the bubble sequence now occur, as duplex DNA, on each side of an axis of symmetry, creating the GAA and TC arms, where the two potential origin sequences differ in length by a single nucleotide. During replication, the dimer junction is resolved into two structures, an "extended" palindromic form, and a "turnaround" form that recreates the left-end hairpin. The extra nucleotide in the GAA arm renders this origin inactive and restricts replication initiation to the TC arm of the dimer junction, causing the resolution process to be predominantly asymmetric (Christensen and Tattersall, 2002).

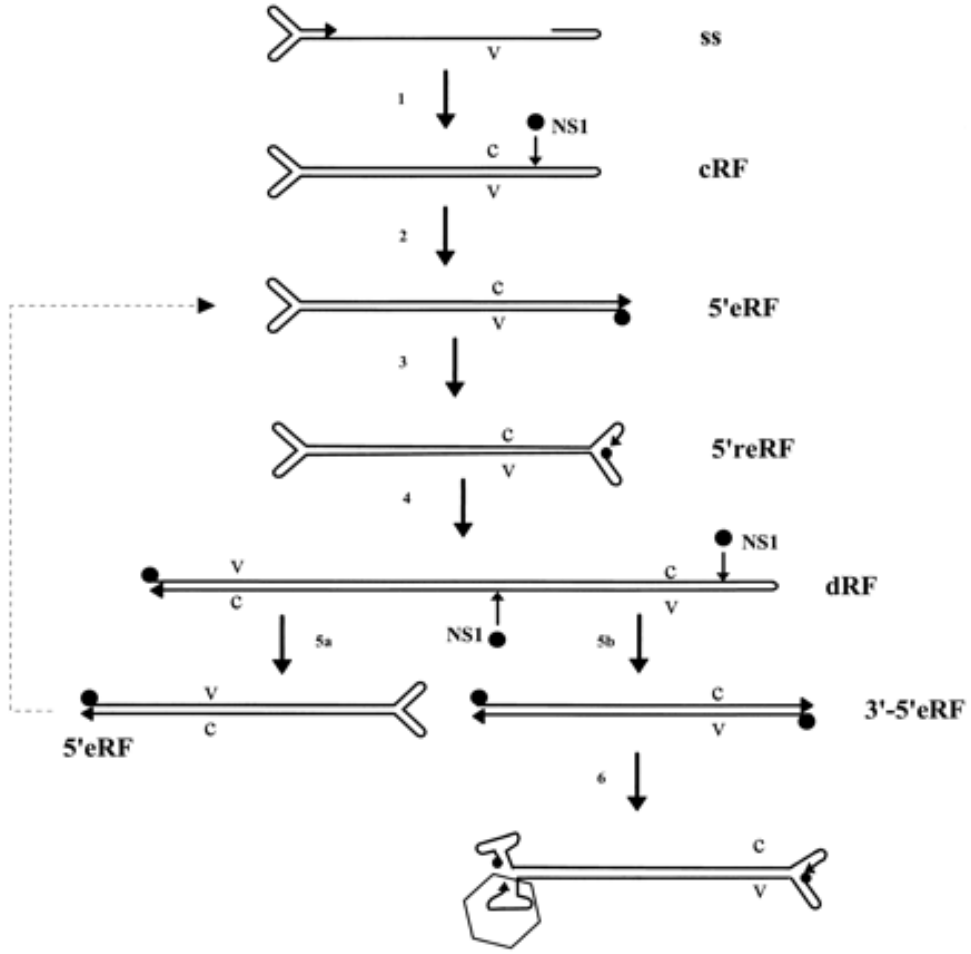


Figure 4. **Modified rolling hairpin model for MVM DNA replication**. NS1 is shown as a small filled circle. Small arrowheads indicate DNA 3' ends. ss, ssDNA; cRF, covalently closed RF; eRF, extended RF; reRF, rabbit-eared RF; v, viral strand; c, complementary strand. The open polygon in step 6 represents the capsid (Reprinted from Kuntz-Simon *et al*, 1999)

Mapping of the minimal sequences necessary for replication of the TC arm has revealed that the origin is approximately 50 bp long and contains three distinct recognition elements: the NS1 cut site; the $(ACCA)_2$ repeat motif, which is a high-affinity NS1 binding site (and acts in an ATP-dependent manner); and a consensus activated transcription factor (ATF) binding site (Figure 5-C). DNase I protection analysis revealed that NS1 extends over a region of approximately 43 nucleotides, protecting sequences surrounding the recognition site, including the nick site (Christensen and Tattersall, 2002; Christensen et al., 1997).

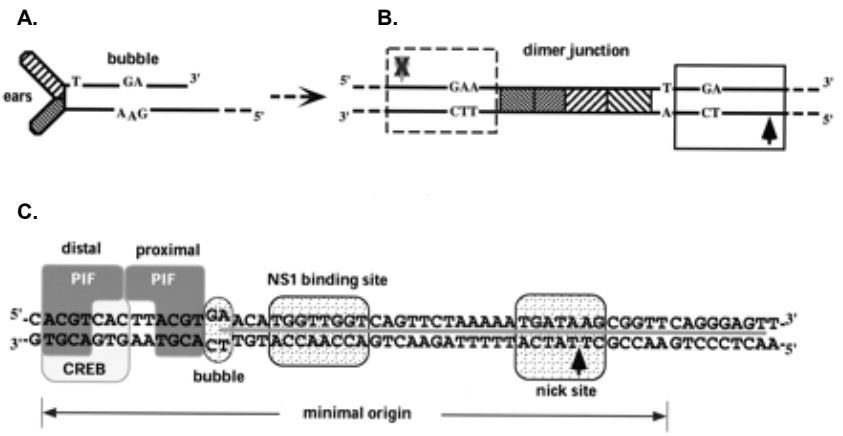


Figure 5**. Formation and organization of the MVM origin of replication**. (**A**) Structure of the left-end hairpin. (**B**) Organization of the left-end hairpin sequences within the duplex dimer junction generated by replication through the hairpin. (**C**) Sequence of the active origin showing the different elements involved in replication. The PIF binding site overlaps a consensus binding site for the CREB/ATF family of host transcription factors. The two tetranucleotide motifs bound by PIF are designated “distal” and “proximal” to indicate their positions relativbe to the NS1 binding site. Other boxes indicate sequences involved in the bubble dinucleotide, the (ACCA)2 NS1 binding site, and the nick site. (Reprinted from Christensen et al., 2002)

While NS1 can bind by itself, it cannot nick the origin because it requires the cooperation of a cellular factor, called Parvovirus Initiation Factor (PIF), which binds to the ATF region of the MVM 3' minimal origin, enabling NS1 to introduce a site-specific, single-strand nick, and leaving the protein covalently attached to the 5' end generated at the nick site. The ATF region recognized by PIF is highly conserved in the 3' hairpin of many autonomous parvoviruses, such as MVM, H-1, LuIII, and to some degree in FPV and CPV (Christensen et al., 1997; Christensen and Tattersall, 2002; Christensen et al., 1997). PIF and NS1 form a high-affinity ternary complex on the TC arm, capable of nicking and subsequently initiating replication at this origin. A model for the replication fork has been proposed (Figure 6), in which the components for the replication of the leading-strand have been identified as proliferating cellular nuclear antigen (PCNA), single-strand DNA binding protein (RPA), δ DNA polymerase (Pol δ), replication factor C, and parvoviral protein NS1. RFC recognizes the primer-template junction, loads PCNA onto the template to form the sliding clamp that stabilizes Pol δ interaction with its template, and promotes processive chain elongation. Pol δ, PCNA, RFC and RPA can be assumed to represent the minimal elements necessary for parvovirus DNA conversion. Some studies point to the involvement of cyclin A in the activation of complementary strand synthesis during S-phase (Bashir et al., 2000; Christensen and Tattersall, 2002; Cossons et al., 1996).

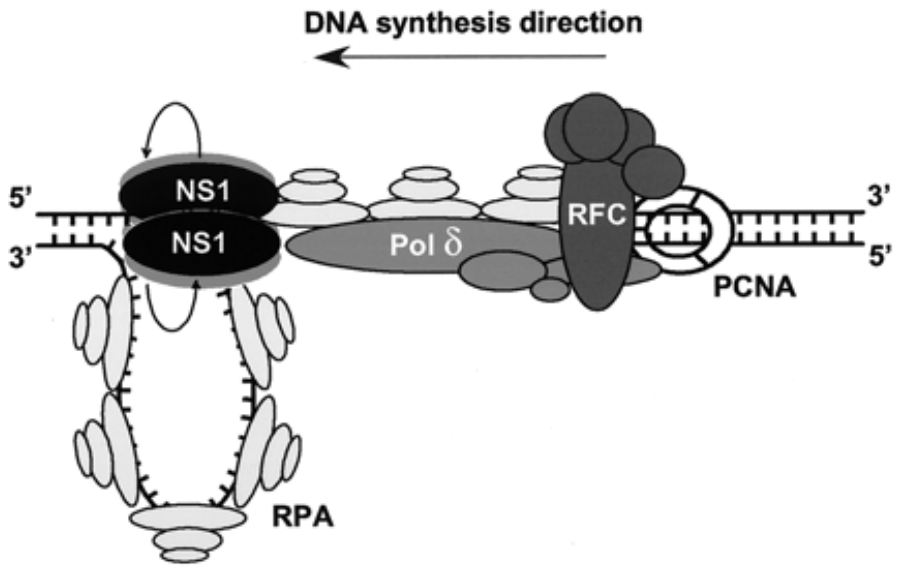


Figure 6. **Proposed model of a minimal parvovirus replication fork**. This model includes various components of the leading-strand synthesis machinery: proliferating cellular nuclear antigen (PCNA), single-stranded DNA binding protein (RPA), the replication factor C (RFC), and polymerase gamma (Pol δ). (Reprinted from Christensen *et al*, 2002).

Replication of Adeno-associated virus (AAV) DNA in vivo occurs by a single-strand displacement mechanism similar to that observed for adenovirus (Ad). Figure 7 illustrates the model for replication starting with linear duplex AAV. The AAV genome contains an inverted terminal repeat (ITR) which is thought to hairpin on itself to serve as the primer to initiate synthesis. When the elongating strand has been synthesized to the end of the template, replication can stop or the newly made strand can fold on itself to initiate a second round of replication. In the latter case, replicative forms would include concatemers of the AAV genome; these have been identified (Ward and berns, 1992; Ward and Berns, 1996). Ends of replicative intermediates are frequently in the hairpinned form, in

which the two complementary strands are covalently cross-linked. Eventually, the cross-link is cleaved by the AAV Rep68/Rep78 (homologous to NS1) protein at a site 124 nucleotides (nt) in from the original 3' end of the template, then the 3' end of the parental strand is resynthesized, using the transferred hairpin sequence as the template. In this manner, the covalent linkage is dissolved, the original 124 nt at the 3' end of the parental template strand are transferred to the 5' end of the progeny strand, and the complete sequence of the ends of the viral genome are maintained. Productive infection by AAV in cell culture normally requires coinfection by a helper virus, usually Ad. In the absence of helper virus, AAV gene expression is repressed and viral DNA synthesis is not detectable. Most of the Ad helper functions which have been defined are primarily involved with the regulation of gene expression. Thus, the available data from in vivo experiments suggest that the failure to detect AAV DNA replication in the absence of Ad coinfection is primarily due to a lack of induction of AAV gene expression (Linden et al., 1996; Ward and Berns, 1996).

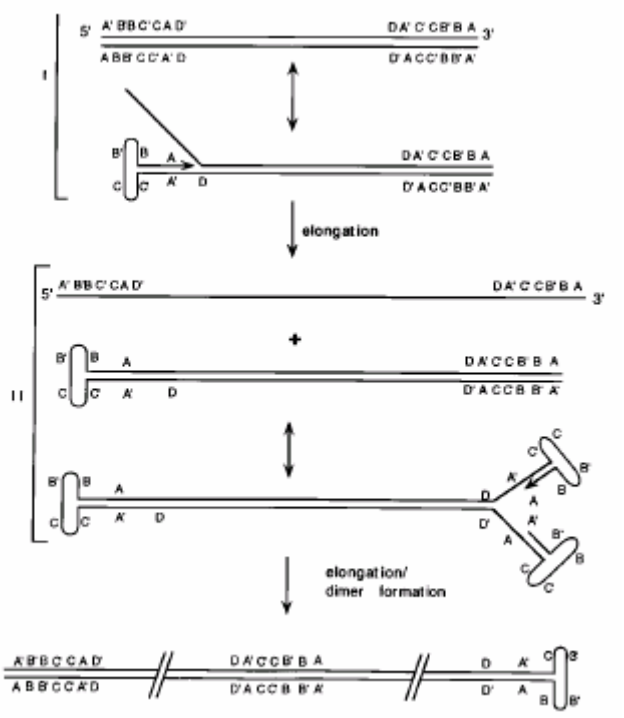


Figure 7. **Model of replication of linear duplex AAV.** The 3' end of one strand folds into a hairpin. This 3' end serves as a primer for single-stranded displacement producing a linear duplex with one hairpinned end and a displaced single-stranded genome. The newly synthesized 3' end on the duplex molecule folds over on itself and acts as a primer for continued single-strand displacement synthesis. The result is a dimer molecule which can be resolved into unit-length linear duplexes by Rep endonuclease nicking at the terminal resolution sequence (Reprinted from Ward and Berns, 1996).

Encapsidation

In addition to the helper virus requirement and terminal sequence characteristics, the strand specificity of viral DNA encapsidation was the basis for classification of parvoviruses as defective or autonomous. The uniqueness of the encapsidation of the minus strand, that which is complementary to mRNA, was believed to be a distinctive feature of the autonomous parvoviruses, in contrast to the defective parvoviruses, which encapsidate strands of both polarities with equal frequency. It was the common believe for some time, that parvoviruses containing identical palindromic sequences at either end of the genome packaged either strand, while parvoviruses containing distinct termini packaged predominantly minus strand (Bates et al, 1984; Berns, 1996).

Table 1. **Encapsidation Pattern of Selected Parvoviruses**

Parvovirus	Terminal hairpin	Encapsidation Pattern
AAV	Identical	50 % (+), 50 % (-)
B19	Identical	50 % (+), 50 % (-)
BPV	Non-identical	15% (+), 85% (-)
H1	Non-identical	99 % (-)
MMV	Non- identical	99 % (-)
LuIII	Non-identical	50 % (+), 50 % (-)

(Adapted from Berns, 1996)

However, further studies demonstrated that the uniqueness of the termini is not the determinant of packaging bias. While the rodent parvoviruses minut virus of mice (MVM), Rat virus, and H-1 have closely related terminal sequences and encapsidate 99% minus strand, bovine parvovirus, lapine parvovirus and the *Aedes densonuleosis* virus encapsidate approximately 10 to 15% of the plus strand. Parvoviruses B19 and LuIII have an encapsidation pattern similar to that of the defective parvovirus AAV, they both encapsidate plus and minus strands with equal frequency (Table 1). In the case of B19 this pattern is expected since the terminal palindromes of this parvovirus, like the defective parvovirus AAV, are identical (Corsini et al., 1995; Diffoot et al., 1989).

Parvovirus LuIII does not sustain this observation because it possesses nonidentical palindromic termini and yet encapsidates both plus and minus strands in a 50% ratio, which implies that identical termini are not necessary for equal encapsidation of strands of both polarities. After comparison of LuIII with rodent parvoviruses MVM and H-1, an 80% overall sequence identity with the genomes of each virus was found, and over 90% of their terminal sequences are identical. They also show similar genomic organization and infect the same type of cells. A closer look to the genome of these parvoviruses exposed two regions of the LuIII sequence that differ in a significant manner from those of MVMp and H-1. Both regions are found downstream from the capsid coding region. There are two copies of a tandem sequence 65-nt long, that appears as a direct repeat at the end of the right ORFs of MVMp and H-1. A sequence comparable to this one is only found once in LuIII. Nevertheless, MVMi also presents a single copy of this sequence and it retains the encapsidation pattern of MVMp and H-1, which leads to believe that the presence of this direct repeat sequence does not influence the viral encapsidation pattern (Diffoot et al., 1993).

The second region where differences can be found is at m.u. 89 of parvovirus LuIII. It is an A-T rich sequence, 6 bases downstream from the end of

the right ORF, unique to LuIII, which was thought to be responsible for the encapsidation pattern that the parvovirus presents. The A-T region is 47 nucleotides long and is not present in either MVM or H-1 (5'-TATGCTCTATGCTTCATATATATTATATATATTATTATACTAACTAA-3').

Studies where the LuIII genome was aligned with that of MVMp showed that the A-T rich region disrupts a sequence near the right palindrome of MVMp, which was previously identified by Tam and Astell to be a *cis*-acting replication signal. Four cellular proteins bind to this region, thought to be involved with the replication of the MVM genome. The interruption of this protein-binding region by the AT sequence might eliminate strand-selective encapsidation and confer the ability to encapsidate either strand. Related studies suggest that the encapsidation pattern of the parvoviruses is a property of their genomes and is not determined by properties of the virion or the nonstructural proteins (Corsini et al, 1995; Diffoot et al, 1989; Difffoot et al., 1993).

Gene Therapy

Gene therapy is a technique for correcting defective genes responsible for disease development. Commonly, an altered gene is associated with different forms of tumors or cancers. Many different approaches have been addressed regarding gene therapy, the most popular being the insertion of a normal gene into the genome to replace an abnormal, disease-causing gene. A carrier molecule called a vector must be used to deliver the therapeutic gene to the patient's target cells. Currently, the most common vector is a virus that has been genetically altered to carry normal human DNA. Several classes of viruses are in use, or are being developed, as gene therapy vectors, among them Retroviruses, Adenoviruses, Herpes Simplex Viruses, and Adeno-associated viruses.

Retroviruses and AAV have a very efficient integration mechanism which should allow long-term expression of transduced genes. Recombinant AAV (rAAV) systems attract enormous interest for use as gene vectors primarily for its unique features such as safety, high titers, broad host range, and vector integration. Recently, rAAV-mediated *in vivo* gene transfers have demonstrated efficient long-term transduction (from 3 months to more than 1.5 years) and lack of cytotoxicity and cellular immune responses, when recombinant molecules of AAV virus with the maker protein β-Gal and the secreted protein erythropoietin were injected in muscle tissue (Kessler et al., 1996; Xiao et al., 1996). Further experients with other tissues, such as muscle, lung, gut, liver, CNS and eye (Monahan and Samulski, 2000) generated near identical results. Other studies show that AAV has the ability to integrate into human chromosome 19 without causing oncogenicity or mutagenesis (Di Pasquale and Stacey, 1998; Maxwell et al., 1996). Hence, AAV seems like an appropriate candidate for long-term gene therapy against genetic defects and cancer.

However, in some situations, long-term persistence may be undesirable and there is a need for small, non-integrating viral vectors. Some of the autonomous replicating parvoviruses, such as H-1, MVM and LuIII, possess properties that make them interesting candidates for gene therapy. For instance, they do not integrate their DNA into cellular chromosomes, they are non-pathogenic to humans, and they are oncotropic and oncolytic in transformed cells. It has been shown that the formation of spontaneous, virally or chemically induced and transplanted tumors is reduced in parvovirus infected animals (Brown et al., 2002; Faisst et al., 1998; Kestler et al., 1994). The oncotropic and oncolytic characteristics of parvoviruses appear to be determined by (1) their enhanced DNA replication capacity in transformed human cells, (2) the oncogene- and cell cycle-dependent activation of the viral early promoter, and (3) the oncogene-induced cytotoxic activity of the non-structural viral protein NS1 (Kestler et al., 1994; Mousset et al., 1998).

Many studies have been conducted with autonomous parvoviruses, especially H-1 and MVM, where they have determined that these viruses are weakly pathogenic to their natural hosts, and that they have a strong predilection for tumor cells. However, although they possess an intrinsic oncosupressive activity, natural parvoviruses are in many instances not potent enough to antagonize tumor development. A therapeutic approach, using autonomous parvoviruses, implies designing recombinant parvovirus-based vectors which (1) can be efficiently packaged into infectious viral particles, (2) retain the parvoviral tropism for tumor cells, and (3) selectively express a potent therapeutic gene, such as one coding for a toxin, a prodrug, or a cytokine, in order to increase the intrinsic parvoviral antineoplastic activity (Dupont et al., 1994; Dupont et al., 2000; Gancberg et al., 2000; Haag et al., 2000).

CHAPTER III
MATERIALS AND METHODS

Construction of pBLu(+) and pBLu(-). An aliquot of 100ng of pBluescript vector (Stratagene, La Jolla, CA) (Figure 7) was digested at 37°C for 1 hour with the restriction enzyme *Bam HI* (Roche, Nutley, NJ) in a total volume of 10μL (1μL pBluescript (100ng), 1μL 10X buffer B, 1μL enzyme (1 U/μL) and 7μL of water). After digestion the vector was precipitated for 30 min at -80°C with 1μL 3M sodium acetate and 2 volumes 100% ethanol. Samples were then centrifuged at 13,000 rpm for 15 min. The vector was resuspended in 10μL TE buffer (10mM Tris-Cl pH 8.0, 1mM EDTA pH 8.0) and treated with the CIAP (Calf Intestine Alkaline Phosphatase) (Roche, Nutley, NJ) enzyme for 30 min, in a reaction of 50μL total volume (10μL vector, 5μL CIAP buffer, 34.7μL water and 0.3μL CIAP[1U/μL]) at 37°C. An additional volume of 0.3μL of CIAP enzyme (1U/μL) was added to the reaction and incubated for 30 min at 37°C. Following CIAP treatment the sample was extracted with phenol/chloroform/isoamyl alcohol (25:24:1)(Fisher Scientific, Fair Lawn, NJ), and precipitated with 5μL 3M sodium acetate and 2 volumes 100% ethanol, at -80°C for 30 min. The DNA was resuspended in 10μL of TE buffer.

To separate the LuIII genome from pUC19, the clone pGLu883ΔXba (1μg/μL) (Figure 8) was treated with the enzyme *Bam HI* at 37°C for 1 hour, in a reaction of a total volume of 10μL (1μL pGLu883Δxba, 1μL 10X buffer B, 1μL enzyme (1 U/μL), and 7μL water). After digestion, the sample was resolved electrophoretically on a 1% agarose gel-1X TBE buffer (89mM Tris base, 89mM boric acid, and 2mM EDTA) at 68 volts for 2 hours. The LuIII fragment (5135 bp) was excised from the gel matrix and extracted with the GeneClean Spin Kit (Qbiogene, Inc., Carlsbad, CA), following manufacturer's instructions.

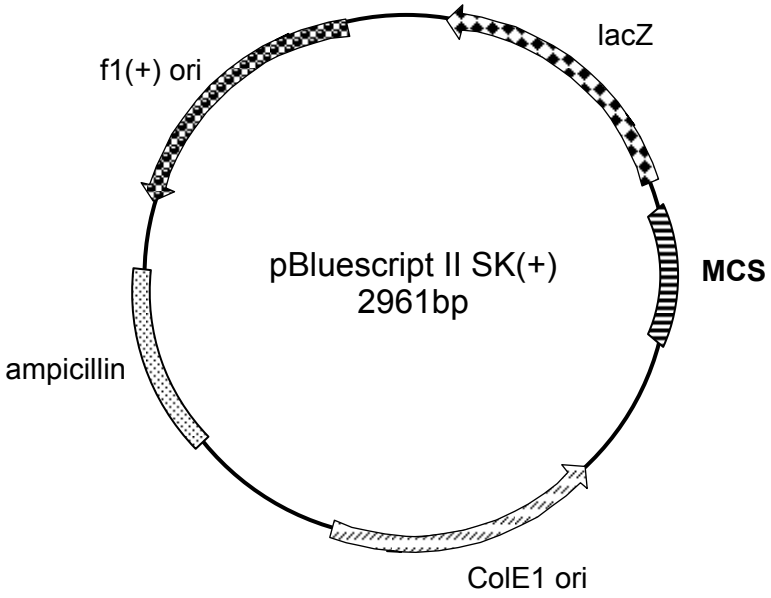


Figure 8. **Genomic map of pBluescript II SK(+)** (Stratagene, La Jolla, CA). The f1(+) ori is the f1 filamentous phage origin of replication. ColE1 ori represents the plasmid origin of replication. LacZ gene. Also shown are the ampicillin selection gene and the multiple cloning site (MCS).

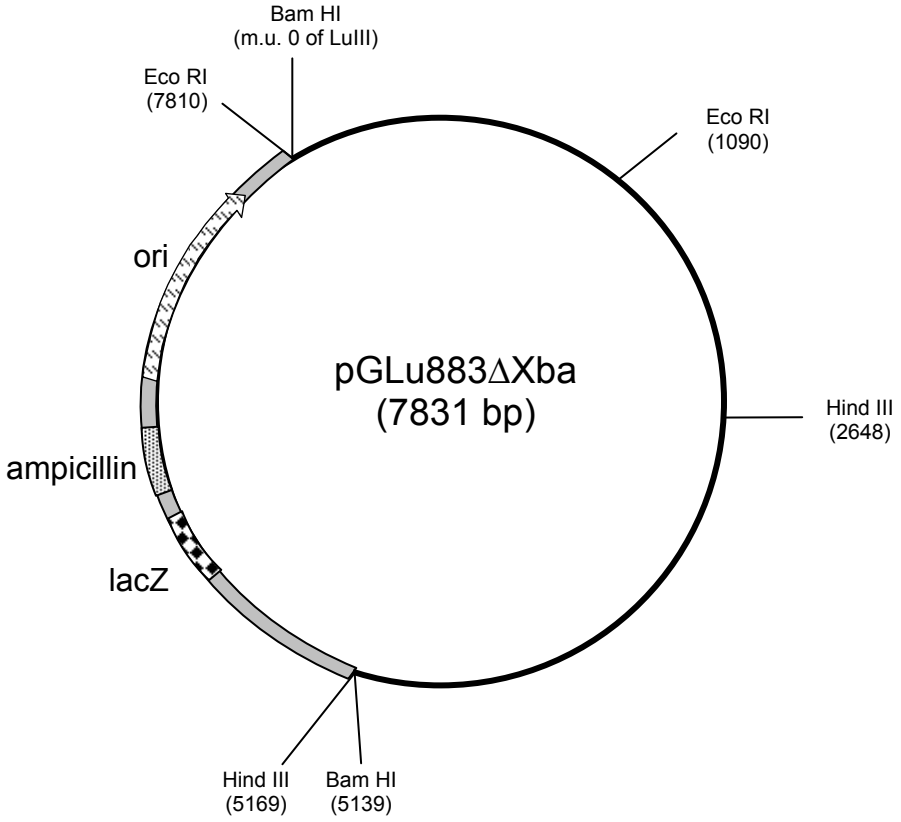


Figure 9. **Genomic map of pGLu883ΔXba** (Maxwell et al.,1993). The pUC19 origin of replication (ori), ampicillin resistance selection gene and lacZ recombinant selection gene are shown. Restriction sites with their respective positions for the enzymes *Bam HI*, *Hind III*, and *Eco RI* are indicated. The LuIII genome is represented by the black line.

Ligation of the pBluescript vector digested with *Bam HI* and the extracted LuIII fragment was performed at 4°C overnight in a total volume reaction of 20μL (1μL pBluescript (20ng), 5μL LuIII genome (100ng), 2μL buffer, 1 μL enzyme (1U/μL), 11μL water) using T4 DNA ligase (Roche, Nutley, NJ). A ligation control reaction, at 4°C in a total volume of 20μL (1μL pBluescript, (20ng), 2μL buffer, 1μL enzyme (1U/μL), 16μL water) was also done.

Bacterial transformation. Competent cells of the *E. coli* XL1-Blue MRF' (Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F´ *proAB lac* ^{q}*Z*Δ*M15* Tn*10* (Tet^{r})] (Stratagene, La Jolla, CA) strain were prepared by inoculating 1 colony in 5μL of LB (Luria broth) media with tetracycline (12.5μg/mL) and grown overnight at 37°C. An aliquot of 1mL was taken and inoculated in 100mL of LB-tetracycline media and subsequently incubated at 37°C for an additional 3 hours. The 100mL culture was split into two 50mL polyethylene tubes and centrifuged at 2500rpm for 10 min at 4°C. The supernatant was discarded; the pellet was gently resuspended in 25mL of cold 50mM $CaCl_2$, and placed in ice for 1 hour. The tubes were centrifuged a second time as described above, the pellet recovered was resuspended in 5mL of cold 50mM $CaCl_2$/20% glycerol, and aliquoted into 100μL samples, cells were placed on ice for an additional hour.

Transformation was performed as follows: four microtubes with 100μL of competent cells each were labeled as shown in Table 2 below.

Table 2. **Transformation of XL1-Blue cells with the product from ligation of pBluscript SK(+) vector and LuIII genome.**

Tube	DNA	DNA Concentration (ng)
Blank	None	0
Standard	Uncut vector (pBluescript II SK(+))	10
Ligation control	pBluescript, CIAP and ligated	20
Experimental	pBluescript + LuIII genome insert	20

After gently mixing DNA with competent cells, they were placed in ice for 30 min, and subsequently submitted to thermal shock at 42°C for 2 min followed by the addition of 100μL LB media. Cells were incubated for 1 hour at 37°C. The total volume of the standard, ligation control and experimental tubes were seeded in LB media plates containing 50μg/mL ampicillin, the contents of the blank tube was seeded in LB plates with and without ampicillin (50% of the total volume on each plate). Each sample was plated with 50μL of X-gal (20mg/mL, dissolved in dimethylformamide). Plates were incubated overnight at 37°C. Transformants were inoculated in 5mL of LB-ampicillin broth, and incubated overnight at 37°C in an orbital shaker at 200 rpm. The plasmid DNA was isolated using the alkaline lysis miniprep purification protocol (small scale plasmid purification) (Ausubel et al., 1994).

Restriction analysis and sequencing. Potential recombinants were submitted to restriction analysis with the enzymes *Bam HI*, *Eco RI*, *Hind III* (Roche, Nutley, NJ), and *Eco RV* (New England Biolabs, Beverly, MA). Digestions were performed in volumes of 10µL (2µl DNA, 1µL 10X buffer, 1µL enzyme (1U/µL) and 6µL water). The samples were resolved electrophoretically at 68V for 4 hours in 1.2% agarose gels-1X TBE buffer. Based on the fragment pattern obtained the pBLu (+) and pBLu (-) constructions were selected.

Selected samples were sequenced at the UMDNJ Molecular Resource Facility, in Newark, NJ, using the Dideoxy DNA sequencing method.

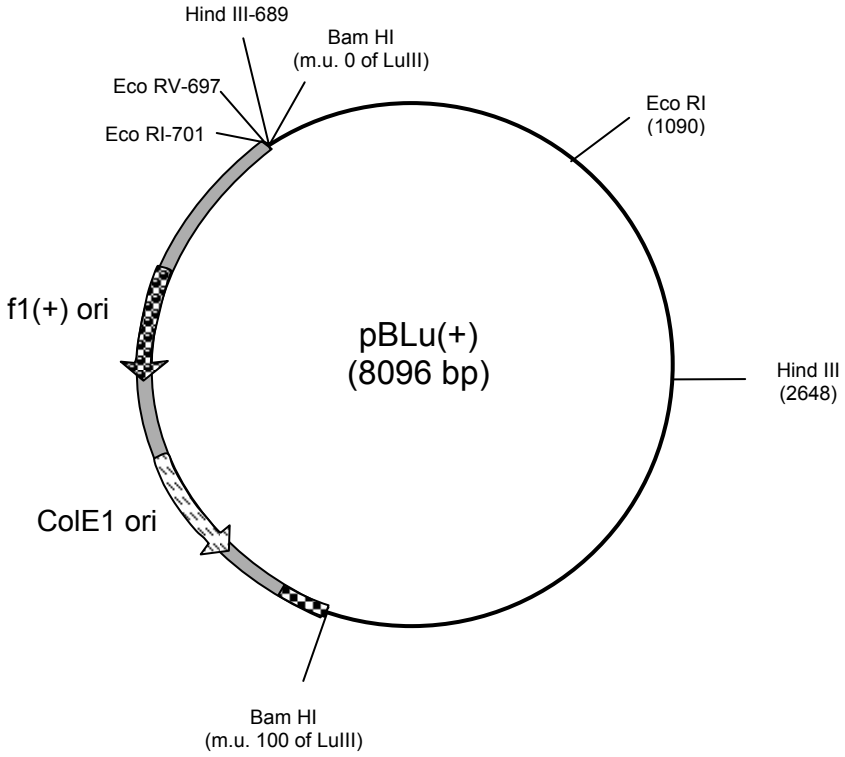


Figure 10. **Genomic map of the pBLu(+) clone.** Restriction sites for the enzymes *Bam HI, Eco RI, Eco RV* and *Hind III* are shown in the LuIII fragment (represented by the black line) and in the vector pBluescript SK(+) multiple cloning site (MCS). ColE1 ori (plasmid origin of replication), f1(+) ori (f1 filamentous phage origin of replication).

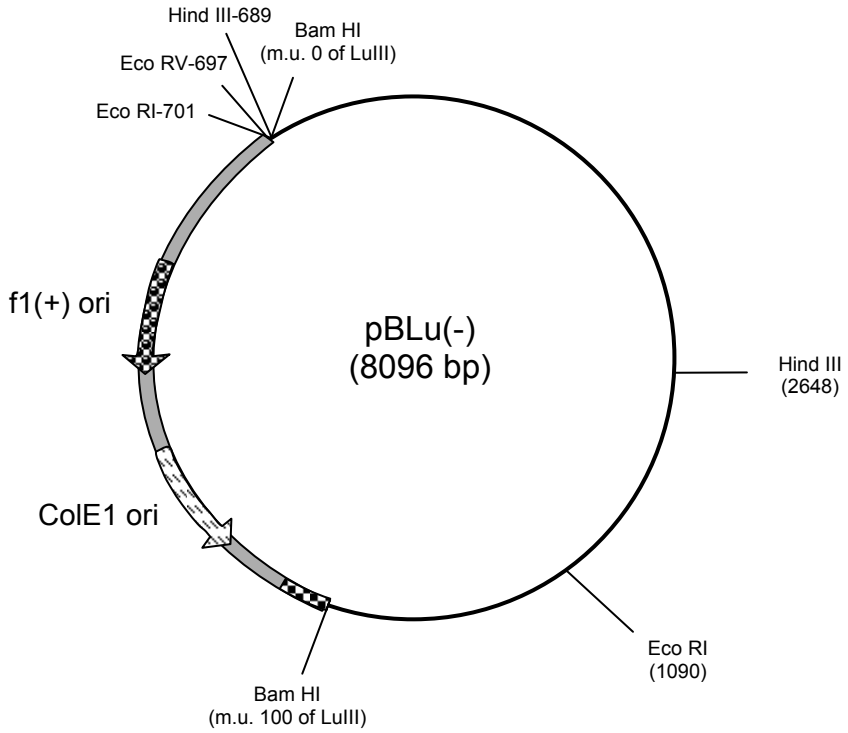


Figure 11. **Genomic map of the pBLu(-) clone.** Restriction sites for the enzymes *Bam HI, Eco RI, Eco RV* and *Hind III* are shown in the LuIII fragment (represented by the black line) and in the vector pBluescript SK(+) multiple cloning site (MCS). ColE1 ori (plasmid origin of replication), f1(+) ori (f1 filamentous phage origin of replication).

Production of single-stranded DNA.

Three different strategies were utilized to obtain Single-stranded (ss) LuIII DNA. Originally the proposed method to obtain ssDNA was the one using a helper phage to aid in the replication of the single strand. After many failed attempts with this method, two other strategies were developed, one using PCR and the other using *Exonuclease III*.

I. Single-stranded DNA generated using a helper phage. A colony of each construct was inoculated in 10mL LB-ampicillin (50µg/mL) media with 10µL of VCM13 phage (10^7-10^8 pfu/mL), the cultures were grown at 37°C with vigorous aeration for 2 hours, after which 70µg/mL of kanamycin were added and the cultures were left overnight at 37°C. A volume of 1.5mL of each culture was centrifuged for 5 min at 13,000rpm. After centrifugation, 1mL of supernatant was recovered and placed in a 2.0mL microtube with 250µL of 30% PEG 1.6M NaCl and placed at 4°C overnight for phage particle precipitation. Purification of single-stranded DNA was performed using the QIAprep Spin M13 kit (QIAGEN Inc., Valencia, CA) as described by the manufacturer.

Southern Blot. DNA obtained from the single-strand rescue assay (ssDNA) was treated with the enzyme *Dpn I* in a total volume reaction of 20µL (10µL DNA, 1µL 10X buffer A, 1µL enzyme (1 U/µL), 8 µL water) at 37°C for 4 hours. The samples were resolved electrophoretically at 68 volts for 4 hours in a 1% agarose gel-1X TBE buffer (89mM Tris base, 89mM boric acid, and 2mM EDTA). The DNA was passively transferred and blotted on to a Zeta Probe nylon membrane (Bio Rad Laboratories, Hercules, CA) as described by Ausubel et al., 1994. The genomic infectious clone pGLu883ΔXba was digested with the restriction enzyme *Bam HI* in a reaction of 10µL (1µL DNA (1µg/µL), 1µL 10X buffer B, 1µL

enzyme (1 U/µL), and 7µL water) at 37°C for 1 hour. After digestion the fragment was resolved electrophoretically at 68 volts for 2 hours on a 1% agarose gel-1X TBE buffer. The LuIII fragment (5135 bp) was extracted following manufacturer's instructions using the GeneClean Spin Kit (Qbiogene, Inc., Carlsbad, CA). The fragment was labeled by random primed labeling using digoxigenin 11-dUTP (Roche, Nutley, NJ) to make the desired probe. Hybridization techniques and detection protocols were performed as instructed by the manufacturer (Roche, Nutley, NJ). Autoradiographic exposures were done at one and twenty-four hours.

Bacterial transformation of XL1-Blue cells to confirm ssDNA production using a helper phage. Competent cells and transformation of *E. coli* XL1-Blue MRF' were performed as described previously. Microtubes containing competent cells were labeled as shown in Table 3.

Table 3. **Transformation of bacterial cells using ssDNA generated with a helper phage.**

Tube	DNA	DNA Concentration (ng)
Blank	None	0
Standard	pBluescript II SK(+)	10
ds(+)	pBLu(+) uncut	10
ds(-)	pBLu(-) uncut	10
ss(+) uncut	single-stranded pBLu(+) uncut	~ 100
ss(+)/Dpn I	single-stranded pBLu(+) digested with Dpn I	~100

Transfection of HeLa cells using DNA obtained from the ss rescue protocol using a helper phage. *Hela* cells were grown in 25 cm^2 plastic culture flasks. The tissue was maintained in culture medium consisting of 90% Minimal Essential Medium 1X (MEM) (ICN, Costa Mesa, CA) with 10% Fetal Bovine Serum (FBS) (Hyclone, Logan, Utah) supplemented with PSG (8mM penicillin G, 3mM streptomycin sulfate, 200mM L-glutamine). The culture medium was buffered to a neutral pH of 7 with 0.75N HCl and 7.5% $NaHCO_3$. Cells were incubated and harvested at a temperature of 37°C until the desired confluence (~90%) was obtained. Cells were treated with Trypsin 1X at 37°C for 5 min and pelleted by low-speed centrifugation (3,700rpm) for 7 min, washed with 10mL PBS 1X (137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.4mM KH_2HPO_4) and centrifuged under the conditions described above. The cells were resuspended in MEM 1X to a final volume of 6.4mL.

The cells were transfected by electroporation and labeled as shown in Table 4. The electroporation protocol was performed using sterile cuvettes with 4-mm electrode separation (Eppendorf, Westbury, NY). A single 75 cm^2 bottle of *HeLa* cells (passage 75) was used to make samples of cell suspension (800µl each) (total of 9 samples). Samples were mixed with 5µg of DNA (20µL) and incubated at 37°C for 5 min, after which they were immediately transferred to a cuvette. The electroporation pulse was delivered using a capacitance discharge machine, Gene Pulser II (Bio Rad, Hercules, CA) with settings, for *HeLa* Cells, of 230V and a capacitance of 950µF. After the pulse was delivered the cells were transferred to a 1.5mL microtube and placed in the dark at room temperature for 45 min. The electroporated samples were immediately transferred to 25 cm^2 flasks containing 3mL warm culture medium and placed in a tissue culture incubator at 37°C and 5% CO_2. Medium was replaced the day after transfection, after sufficient time for reattachment of the cells, with 3mL of fresh medium. The flasks were moved to an incubator without CO_2 until 100% cell lysis (CPE-cytopathic effects) was observed.

Table 4. **Electroporation of *HeLa* cells using ssDNA generated using a helper phage.**

25cm^3 Flask	**DNA**	**DNA Concentration (µg)**
Control	None	0
Positive control	pGLu883ΔXba	~5
ss(+) uncut	sspBLu(+) uncut	~5
ss(+)/Dpn I	sspBLu(+) digested with Dpn I	~5

II. Single-stranded DNA generated by PCR. The pBLu(+) construction was digested with the enzyme *Pvu I* (Roche, Nutley, NJ) in a total volume reaction of 10 µL (5 µL DNA, 1 µL 10X buffer H, 1 µL enzyme (1 U/µL) and 3 µL water) at 37°C for 1 hour. After digestion the DNA was precipitated for 1 hour at 80°C in 10% sodium acetate and two volumes of 100% ethanol, and resuspended in 10 µL of water, to use as a template for a PCR reaction. A total number of 4 PCR reactions were performed as depicted in Table 5, using the AccuTaq LA DNA Polymerase Kit from Sigma (St. Louis, MO) and the universal primers Forward and Reverse (Promega, Madison, WI)(Figure 12).

The Perkin Elmer 2400 Thermocycler was used. After 10 min at 98°C for denaturizing, 30 amplification cycles were performed (30 sec at 94°C + 3 min at 72°C), and a final synthesis step of 10 min at 72°C. For analysis, 10 µL of each sample were resolved in a 1.2% agarose gel using TAE 1X buffer, at 72V for 4 hours. The 2 Log DNA Ladder (BioLabs, Inc.) was used as a marker.

Table 5. **PCR Reactions performed to obtain ssDNA using the pBLu(+).**

	1 Negative control	2 (-) strand progeny	3 (+) strand progeny	4 Positive control
Template	0.5 µL	0.5 µL	0.5 µL	0.5 µL
Primers	No primers	3.0 µL forward	3.0 µL reverse	1.5 µL each primer
dNTP's	2.5 µL	2.5 µL	2.5 µL	2.5 µL
Buffer	10.0 µL	10.0 µL	10.0 µL	10.0 µL
ddH_2O	86.5 µL	83.5 µL	83.5 µL	83.5 µL
Taq Polymerase	0.5 µL	0.5 µL	0.5 µL	0.5 µL
Total	100.0 µl	100.0 µl	100.0 µl	100.0 µl

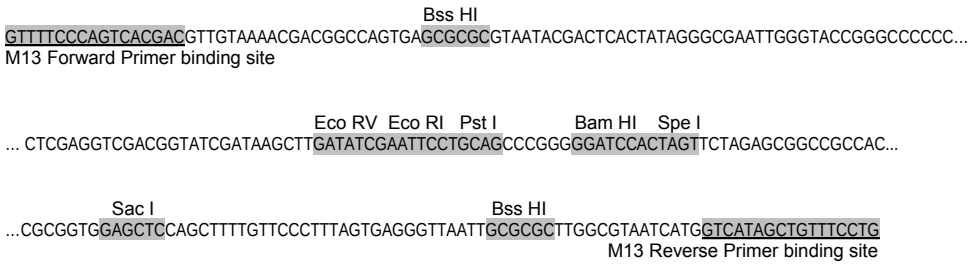


Figure 12. **Location of the Forward and Reverse primers in the pBluescript SK(+) II plasmid.** Cutting sites of relevant enzymes are shown.

III. Single-Stranded DNA generated by Exonuclease III treatment. The pBLu(+) construct was digested with two different sets of enzymes, at 37°C for 24 hours. Each reaction had a total volume of 20 µL, consisting of 10 µL DNA and 6 µL water. For the first reaction 2 µL buffer A and 1 µL each enzyme, *Eco RV* and *Sac I* completed the total volume; for the second one 2 µL buffer H and 1 µL each of *Pst I* and *Spe I* (All enzymes from Roche, Nutley, NJ). After digestion the DNA was precipitated in 2 volumes of Isopropyl alcohol at 80°C for 1 hour, and resuspended in 20 µL of ddH_2O. The resuspended DNA was submitted to treatment with the *Exonuclease III* (*Exo III*) enzyme, for 15 min at 37°C, (Promega, Madison, WI) in a total volume reaction of 50 µL (20 µL DNA, 5 µL Exo buffer 10X, 3 µL enzyme [8 U/µL], and 22 µL water). The reaction was stopped with 10 µL 0.2M EDTA. The DNA was again precipitated following the conditions described above, and resuspended in 20 µL of water. A digestion with the enzyme *Dpn I* was performed using 10 µL of the treated DNA, 1 µL enzyme, 2 µL buffer A, and 7 µL ddH_2O, at 37°C for 4 hours. Samples were resolved in a 1% agarose gel, using TAE 1X, at 72V for 2 hours.

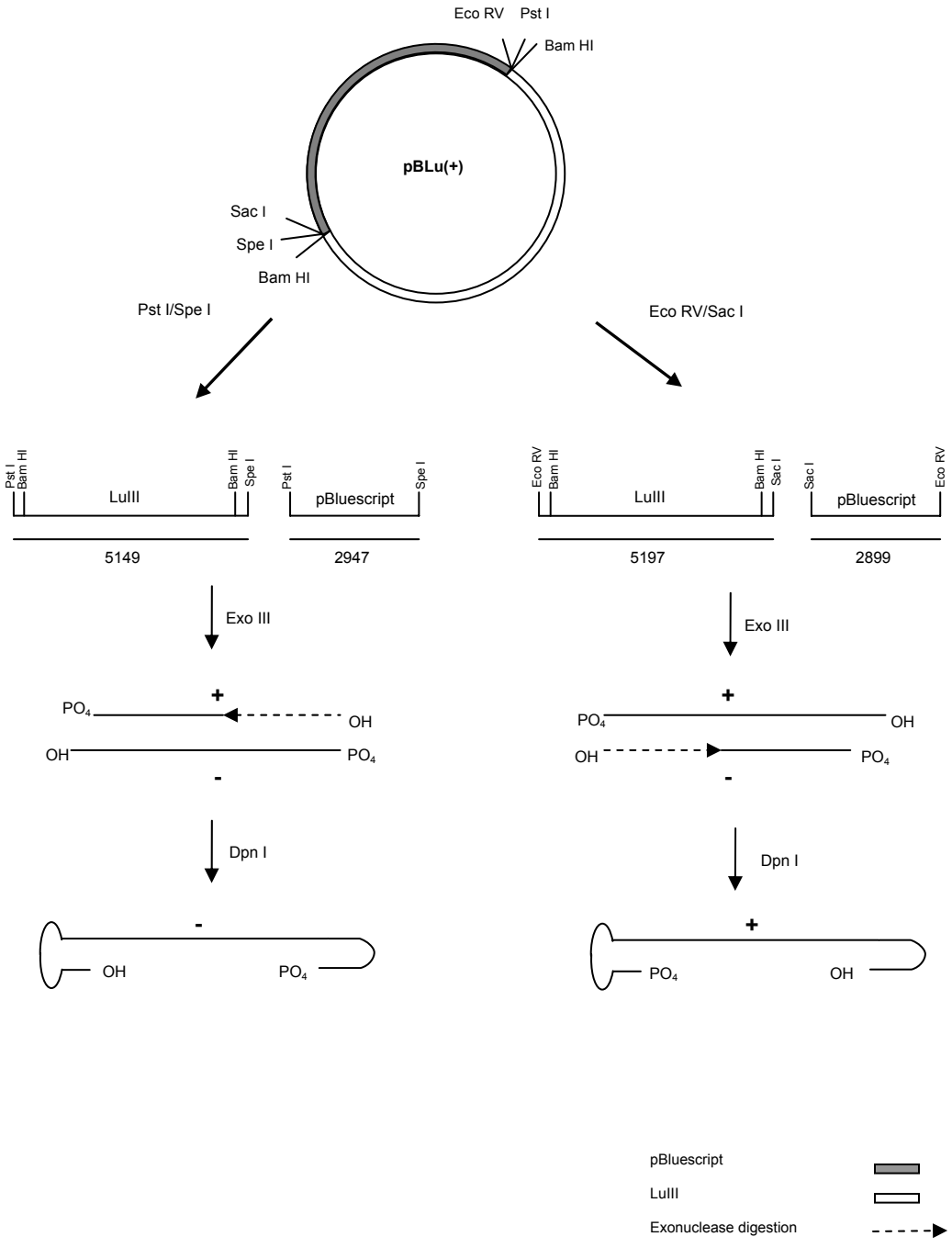


Figure 13. **Generation of LuIII ssDNA using *Exonuclease III.***

Bacterial transformation of XL1-Blue cells to confirm ssDNA production using Exonuclease III. Another set of competent cells and subsequent transformation of *E. coli* XL1-Blue MRF' were performed, this time with DNA treated with *Exonuclease III*. Microtubes containing competent cells were labeled as shown in Table 6 below.

Table 6. **Transformation of bacterial cells with LuIII ssDNA generated with *Exonuclease III*.**

Tube	DNA	DNA Concentration (ng)
Blank	None	0
Standard	pBluescript II SK(+)	10
ds(+)	pBLu(+) uncut	10
(+)/E+S	pBLu(+) cut with Eco RV and Sac I	~100
(+)/P+S	pBLu(+) cut with Pst I and Spe I	~ 100
(+)/E+S+E	pBLu(+) cut with E+S and treated with Exo III	~100
(+)/P+S+E	pBLu(+) cut with P+S and treated with Exo III	~100
(+)/E+S+E+D	ssBLu(+) digested with Dpn I	~100
(+)/P+S+E+D	ssBLu(+) digested with Dpn I	~100
(+)/E+S+E+D P+S+E+D	sspBLu(+) of both polarities	~100

Transfection of HeLa cells using DNA obtained from Exonuclease III treatment. The electroporation of HeLa cells was again performed as described above, but this time with the product yielded from the treatment of pBLu(+) with *Exonuclease III* (Table 7).

Table 7. **Electroporation of *HeLa* cells with LuIII ssDNA generated with *Exonuclease III*.**

25cm^3 Flask	**DNA**	**DNA Concentration (µg)**
Control	None	0
Positive control	pGLu883ΔXba	5
ds(+) uncut	dspBLu(+) uncut	~5
ds(+)/Bam HI	dspBLu(+) digested with Bam HI	~5
ss(+) uncut	pBLu(+)/E+S+E	~5
ss(-) uncut	pBLu(+)/P+S+E	~5
ss(+)/Dpn I	sspBLu(+) digested with Dpn I	~5
ss(-)/Dpn I	sspBLu(-) digested with Dpn I	~5
ss(+) + ss(-)	pBLu(+) of both polarities digested with Dpn I	~5

Rescue of viral particles from transfected cells and infection of HeLa cells. The transfected cells that showed 100% CPE were lysed and virus released by three rounds of freezing and thawing. The cells suspension was transferred to a 50mL polypropylene tube and centrifuged for 10 min at 3700rpm, the supernatant containing the viral progeny was stored at –20°C and the cell pellet was discarded. The viral stock preparation was used to infect *HeLa* cells. The cells were washed twice with 1X MEM to remove traces of serum, and 500µL of the viral stock were added to each flask, and incubated for one hour at 37°C. After

incubation the medium was discarded and replaced by 5mL of 90% 1X MEM/ 10% FBS. Cells were observed until they reached 100% CPE.

CHAPTER IV
RESULTS AND DISCUSSION

Autonomous parvoviruses (APVs) are small animal viruses containing linear single-stranded DNA genomes with palindromic termini. The single strand encapsidated by parvoviruses can be of either plus or minus polarity and the ratio varies among members of the Parvoviridae family (Table 1, Chapter I) (Diffoot et al., 1993). Most of what is known about replication of autonomous parvoviruses comes from studies made with MVM and H-1, which resemble LuIII in over 90% of their genomic sequence. It has been demonstrated that LuIII encapsidates 50% of each strand, similar to AAV and B19; this is in contrast with MVM and H-1 that encapsidates only strands of minus polarity (Bates et al., 1984). A replication model that results in the LuIII encapsidation pattern has not been elucidated. The potential of this parvovirus for use as a gene transfer vector is very promising; it is known to infect human cells and a highly infectious genomic clone of LuIII, pGLu883, is available.

The goal of this project was to study the capability of the plus and the minus strand of parvovirus LuIII to independently establish an infection after transfection into HeLa cells.

Construction of pBLu(+) and pBLu(-). With the purpose of generating only single-stranded DNA the plasmid pBluescript II SK(+) (Stratagene, La Jolla, CA) (Figure 8) was selected as a cloning vector for the LuIII genome. This plasmid can be rescued as single-stranded (ss) DNA, because it contains the f1(+) origin of replication (M13 related), which allows the recovery of the sense strand (with respect to the origin of replication of the plasmid) when co-infected with a helper phage. The plasmid, pBluescript II SK(+), digested with the enzyme *Bam HI* (linear fragment of 2961 bp), and the LuIII genome extracted from the infectious

clone pGLu883ΔXba (linear molecule of 5135 bp) (Figure 14) were ligated. For this reaction the control was pBluescript II SK(+) ligated with itself. Of this ligation two different clones were expected, one with the LuIII fragment inserted in the 0-100 m.u. direction (given by the orientation of the origin of replication), and the other one inserted in the 100-0 m.u. direction (Figures 10 and 11, Chapter III), named pBLu(+) and pBLu (-) respectively.

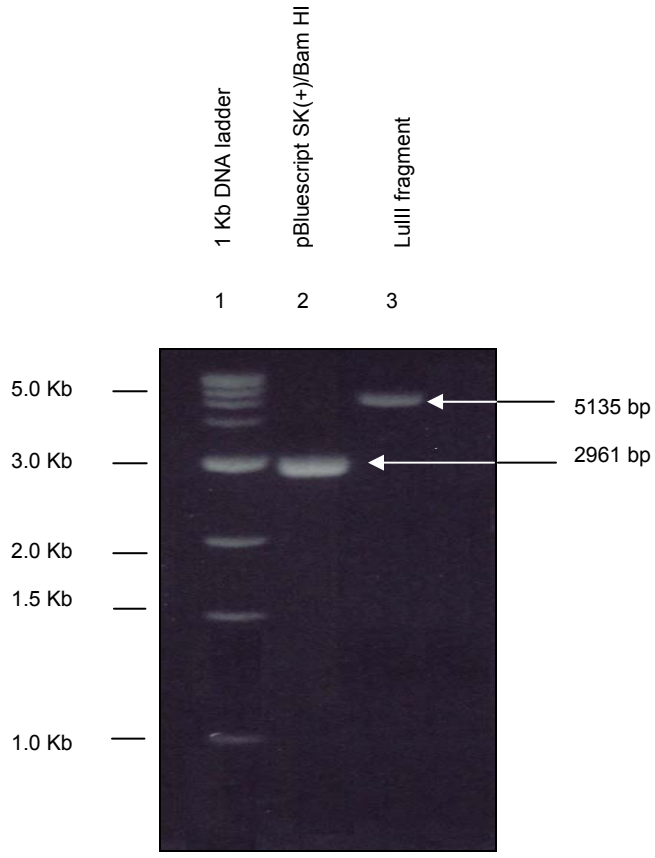


Figure 14. **Parvovirus LuIII full length genome and pBluescript vector fragments used for ligation.** Electrophoresis was performed on a 1.2% agarose gel in 1X TAE buffer at 75V. Sizes of the 1Kb DNA ladder (lane 1) (Biolabs, Inc.) are indicated.

Bacterial transformation. Possible recombinant molecules were transformed into *E.coli* XL1-Blue MRF' competent cells. Results are shown in Table 8 below. The controls used for the transformation resulted in: the blank plate without ampicillin (amp) showed a bacterial lawn, which demonstrates that the cells were still viable after being subjected to the calcium chloride treatment (for competence) and the heat shock (for transformation). The blank plate with ampicillin showed no growth, demonstrating that the antibiotic, and hence the selection, were working properly, and that the cells were sensitive to ampicillin. The transformation efficiency, determined from the standard, was 6.6×10^5 cfu/µg. 46 individual recombinant colonies were obtained. These colonies were inoculated in LB-amp broth, and small scale plasmid preparations were made as described by Ausubel et al. (1994).

Table 8. **Transformation of *E. coli* XL1-Blue with possible pBLu(+) and pBLu (-) constructs**

SAMPLE	DNA concentration	# of colonies
Blank (without ampicillin)	0	Bacterial lawn
Blank (with ampicillin)	0	0
Standard	10 ng	TNTC*
Ligation control	20 ng	0
Experimental	36 ng	46

*Too Numerous To Count

Restriction analysis and sequencing. The minipreps were digested with the enzymes *Bam HI*, *Eco RI*, *Eco RV*, and *HindIII*, and analyzed by DNA electrophoresis. The *Bam HI* digestion liberates the LuIII genome from the vector, generating two fragments, a 2961 bp fragment that corresponds to the vector, and the 5135 bp fragment of LuIII. *Eco RV* was used to confirm that the LuIII fragment was cloned in the correct vector, this enzyme is a non-cutter for pUC19 (the vector in pGLu883ΔXba), and cuts once in pBluescript II SK(+) at nucleotide 697, resulting in a linear molecule of 8096 bp. *Eco RI* was used to determine the orientation of the LuIII fragment in the plasmid, this enzyme cuts once in the vector (nt 701), and once in LuIII (nt 1090). For the construct pBLu(+), one fragment corresponding to two molecules of 4034 bp and 4062 bp are expected. For pBLu(-), two fragments of 1108 bp and 6988 bp are expected. The enzyme *Hind III* has one cut site in the MCS of the vector (nt 689), and cuts once in LuIII at nt 2648; for each construct two fragments are expected when digested with this enzyme. For pBLu(-) these sizes are 2678 bp and 5418 bp (Figure 17), and for pBLu(+) of 2517 bp and 5579 bp (Figure 16).

Of the 46 colonies obtained, 28 presented the pattern expected for pBLu(+), 3 presented the pattern for pBLu(-), and 15 presented a restriction pattern corresponding to the recircularized vector.

Clones with the desired restriction pattern were selected (Figures 15-17). In Figure 15 digestions of pBLu(+) with *Bam HI, Eco RI,*and *Eco RV*, in comparison to pBluescript and pGLu883ΔXba can be seen. The desired orientation generated by the enzyme *Eco RI* can be observed. The expected fragments can be appreciated in Figure 16 when the constructs were digested with *Hind III*. Confirmation of pBLu(-) can be observed in Figure 17, digestions of this construct with the enzymes mentioned above resulted in fragments of the expected sizes.

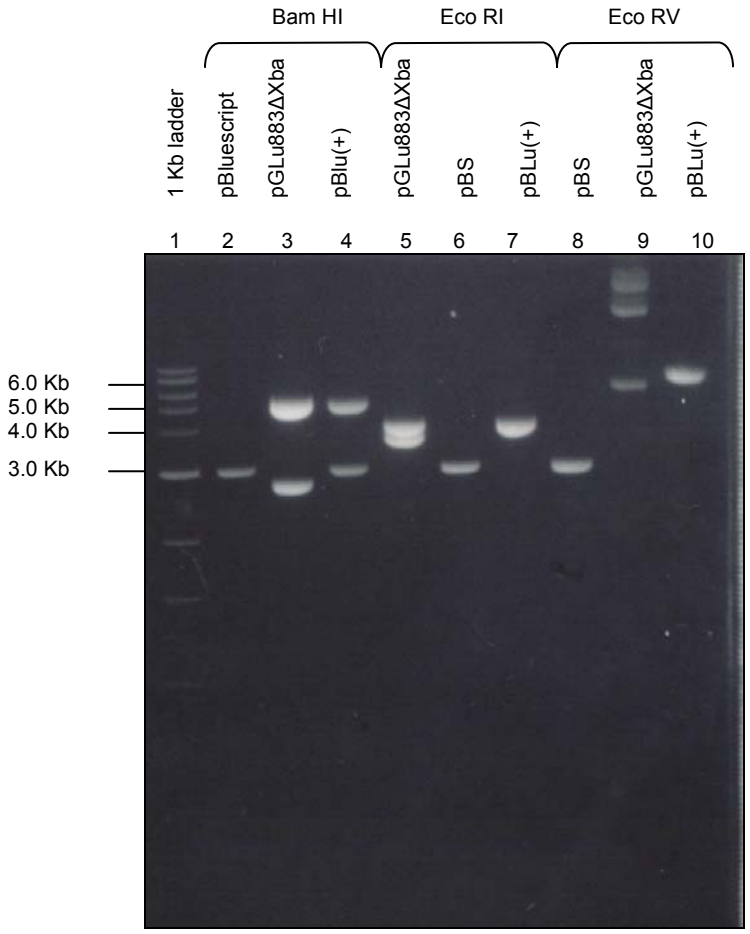


Figure 15. **Digestions of the pBLu(+) construct, pGLu883ΔXba and pBluescript SK(+) II.** Digestions were performed with the enzymes *Bam HI*, *Eco RI*, and *EcoRV*, and electrophoresed on a 1.2% agarose gel in 1X TAE buffer at 75V. Sizes of the 1Kb DNA ladder (lane 1) (Biolabs, Inc.) are indicated.

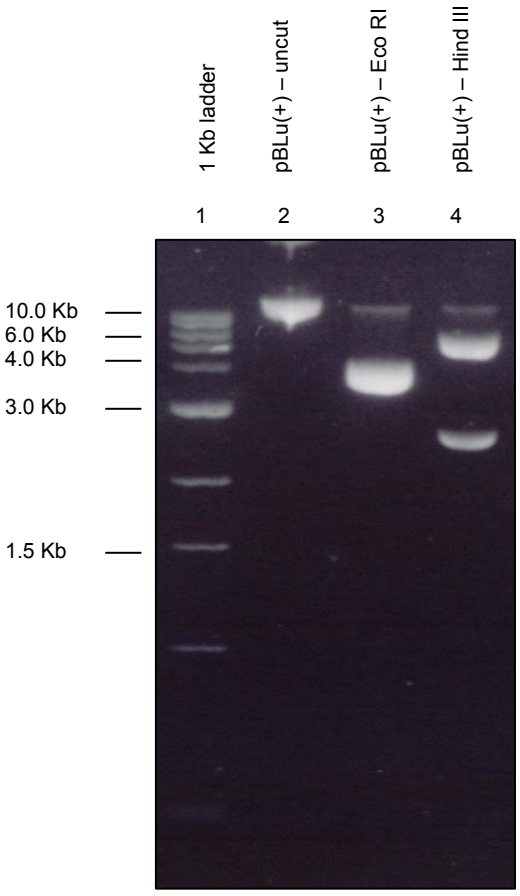


Figure 16. **Digestions of possible pBLu(+) construct with *HindIII*.** Digestions were electrophoresed on a 1.2% agarose gel in 1X TAE buffer at 75V. Sizes of the 1Kb DNA ladder (lane 1) (Biolabs, Inc.) are indicated.

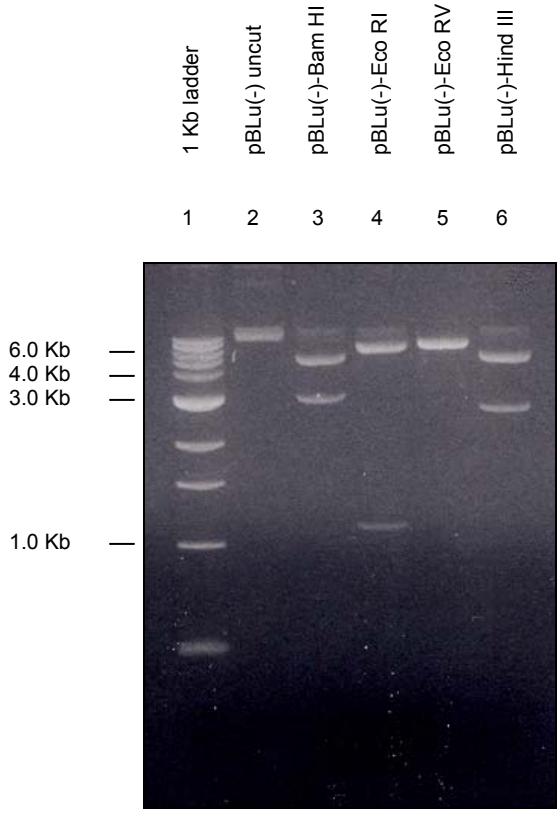


Figure 17. **Digestions of possible pBLu(-) construct**. Digestions were performed with the enzymes *Bam HI*, *Eco RI*, *Eco RV*, and *HindIII* and electrophoresed on a 1.2% agarose gel in 1X TAE buffer at 75V. Sizes of the 1 Kb DNA ladder (lane 1) (Biolabs, Inc.) are indicated.

Further confirmation of these constructs was obtained by sequencing with the universal primers Forward and Reverse, using the Dideoxy DNA sequencing method at the UMDNJ Molecular Resource Facility, in Newark, NJ. Integrity of the termini was confirmed, these needed to be intact for efficient replication and encapsidation of the virus (Corsini et al., 1997; Cotmore and Tattersall, 1992; Tam and Astell, 1993). The Forward primer reads the 3'-termini of pBLu(+) and the 5'-termini of pBLu(-), and the Reverse primer reads the 5'-termini of pBLu(+) and the 3'-termini of pBLu(-). For both constructs the *Bam HI* site, where the LuIII genome was cloned, and the sequence at the 3'-termini were complete (Table 6). However, sequencing of the 5'-termini of both constructs was not possible, most likely due to the secondary structures formed at this terminus.

According to restriction pattern and sequencing of the 3'-termini, two recombinant molecules were selected as the pBLu(+) and pBLu(-) clones, to be used for ss rescue protocols and infection of cancerous cells.

Template: pBLu(+) Primer: M13 Forward

AGGGCGAATTGGGtACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCG
Sma I Bam HI 3'-Termini sequence
AATTCCTGCAGCCCGGGGGATCCCCATCATTTTTAGAACTAACCAACCATGTTCACGTAAG
TGACGTGATGACGCGCGCTCNCNCNCTGCCTTCGGCAGTCACACGTCACTTACGTCTCAC
ATGGTTGGTTAGTTCTAAAAATGATAAGCGGTTCAGGGAGTTTAAACCAAGGCGCGAAAAG
GAAGTCNNTCTTgGtTTTAAGTATATAAGCGACAGCTTAAGTCAGTTACTTACTCTTTCGCTT
ATTCTGtAAGtCGAGACACACAGAGTAACCAACTAACCAACTAGCCATGGCTGGAAACGCG
TACTCTGATGAAGtTTTGGGAACAACTAACTGGtTGAAGGATAA

Template: pBLu(-) Primer: M13 Forward

GACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAA
Sma I Bam HI
GCTTGATATCGAATTCCTGCAGCCCGGGGGATCCCCCCATTAGTATCAATATGTTTTN
Stopped

Template: pBLu(+) Primer: M13 Reverse

CTGATTACGCCAAGCGCGCaATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCAC
Spe I Bam HI
CGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCCATTAGTATCAATATGTTTTT
Stopped

Template: pBLu(-) Primer: M13 Reverse

CaTGATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACC
Spe I Bam HI 3'-Termini sequence
GCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCATCATTTTTAGAACTAACCAACCATGT
TCACGTAAGTGACGTGATGACGCGCGCTACNCNCGCTGCCTTCGGCAGTCACACGTCACT
TACGTCTCACATGGTTGGTTAGTTCTAAAAATGATAAGCGGTTCAGGGAGTTTAAACCAAGG
CGCGAAAAGGAAGTGGGCGTGGTTTTAAGTATATAAGCGACAGAtTTAAGTCAGTTACTTAC
TCTTTCGCTTATTCTGTAAGTCGAGACACACAGAGTAACCAACTAACCAACTAGCCATGGCT
GGAAACGCGTACTCTGATGAAGTTTTGGGAACAACTAACTGGTTGAAGGATAAGAGCAACC
AGGAAGTATTCTCATTTGTTTTTAAAAATGA

Figure 18. **Sequence results for pBLu(+) and pBLu(-) constructs.** The sequence for the 3'-termini and relevant enzymes are highlighted.

Production of ssDNA:

I. ssDNA generated using a helper phage. The ss rescue protocol was performed using the helper phage VCSM13, which has the advantage of conferring Kanamycin resistance to bacterial cells when infecting for ssDNA replication. The pBluescript SK(+) plasmid without the insertion was used as both positive and negative controls. For the positive control the plasmid was inoculated with the helper phage, whereas for the negative control it was inoculated without the helper phage. After incubation, the positive control and the flask inoculated with pBLu(+) showed cell growth, but no growth was observed for the negative control and pBLu(-). Both controls behaved as expected, meaning that the VCSM13 helper phage and the kanamycin where both working properly, the protocol was repeated several times, and an assay using different concentrations of the helper phage and of kanamycin gave the same results. Why pBLu(-) could not produce ssDNA with the helper phage cannot be explained. The three clones available that showed the 100-0 m.u. orientation were repeatedly submitted to restriction enzyme analysis with *Bam HI*, *Eco RI*, *Eco RV, and Hind III*, and all showed the expected fragments for this construction. Also the three samples were used for the ss rescue protocol, and no positive results were obtained. Trying to find an explanation for these results the cloning procedure was repeated using pBLu(+) and pGLu883ΔXba. The samples were submitted to *Bam HI* digestion and later to ligation with T4 DNA ligase. A total of 80 colonies, from 4 independent transformations, were analyzed trying to find clones in the 100-0 m.u. orientation. None of the samples presented the desired orientation. Only 3 of 46 transformed colonies presented the desired restriction pattern for pBLu(-) (Table 8), suggesting that this orientation is not favored, which could explain why the construction did not seem to generate ssDNA when inoculated with the helper phage.

The product obtained from the replication of pBLu(+) with the helper phage was used for further analysis to assess if indeed sspBLu(+) was produced, and if so, if the product was capable of causing infection in HeLa cells.

Southern Blot. The sspBLu(+) was digested with the enzyme *Dpn I* to digest any dspBLu(+) that might still be left in the samples. All the samples were resolved in an agarose gel and then transferred to a nylon membrane. After hybridization with the LuIII probe and radiographic exposure, whereas fragments corresponding to dspBLu(+) and pGLu883ΔXba were observed for the negative and positive controls no fragments could be appreciated (Figure 19). In the case of sspBLu(+), a large fragment of approximately 8 Kb can be seen in the uncut sample lane (Figure 19), and the fragments from the digestion with *Dpn I* can be observed in the lane labeled as sspBLu(+)/Dpn I. The ~4 Kb fragment expected for the ssDNA could not be observed.

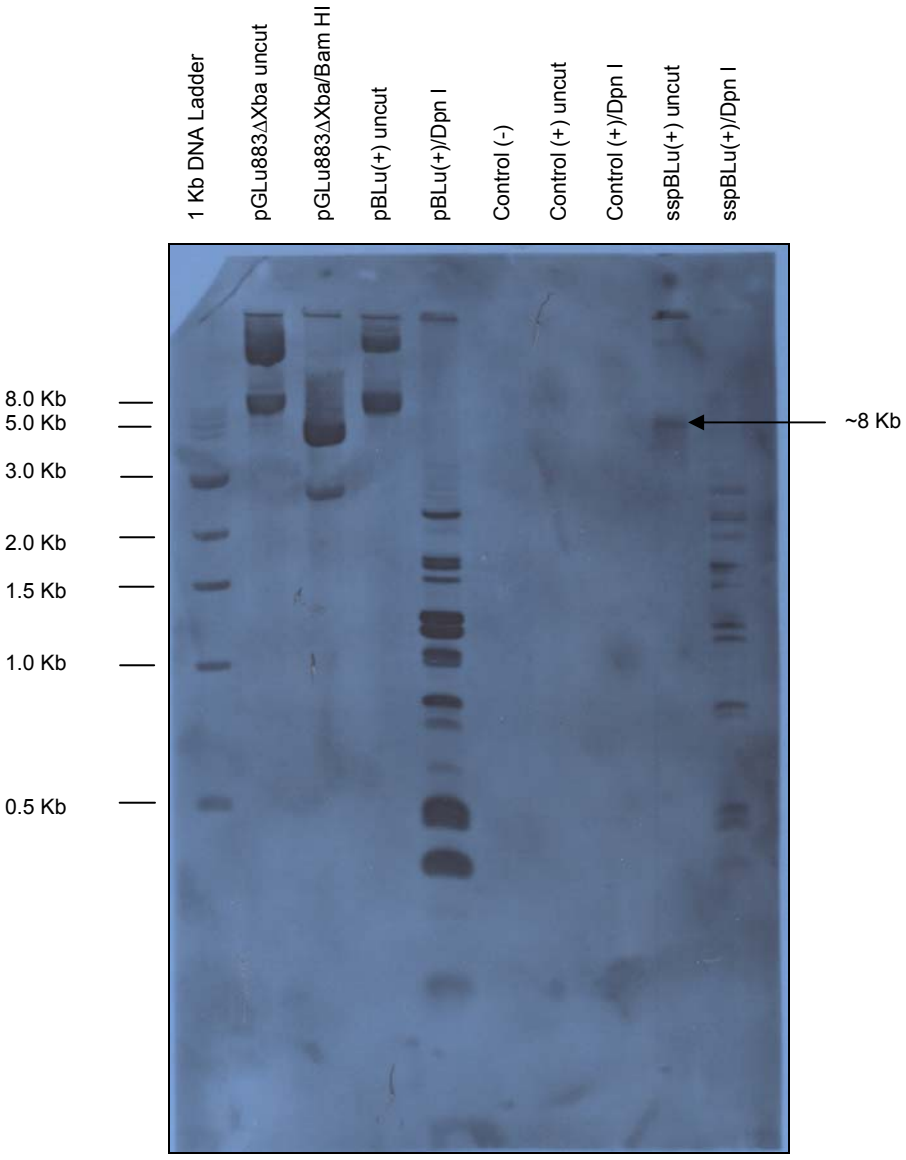


Figure 19. **Southern blot analysis of DNA obtained from the ss rescue protocol using a helper phage.** Sizes of the 1 Kb DNA ladder are indicated. Samples were hybridized with a LuIII probe at 55°C. Overnight exposure.

Bacterial transformation of XL1-Blue cells to confirm ssDNA production using a helper phage. The same DNA that was submitted to southern blot analysis was used to transform bacterial cells, to confirm that no double stranded circular DNA still remained in the samples. As expected, the transformation yielded no colony forming units on the plates transformed with DNA digested with *Dpn I* (Table 9).

Table 9. **Transformation of bacterial cells using ssDNA generated with a helper phage.**

Sample	DNA	cfu/µL
Blank with Amp	None	No growth
Blank w/o Amp	None	Bacterial lawn
Standard	pBluescript II SK(+)	TNTC
ds(+)	pBLu(+) uncut	10
ss(+) uncut	single-stranded pBLu(+) uncut	42
ss(+)/Dpn I	single-stranded pBLu(+) digested with Dpn I	0

Transfection of HeLa cells using DNA obtained from the ss rescue protocol using a helper phage. Evidence of the presence of sspBLu(+) in the samples could not be obtained with the southern blot analysis. Nevertheless, the results of the southern blot and the transformation suggest that no dsDNA is present either. The fact that the fragment corresponding to the ssDNA could not be observed can be attributed to the low concentration of DNA present in the samples. The samples were used to transfect HeLa cells, if any cytopathic effects (CPE) were observed, these were likely due to the sspBLu(+).

The electroporated samples were kept at 37°C for 8 days, during this time the controls behaved as expected. The flask where the cells were not exposed to the recombinant DNA formed a very thick tissue layer, and remained as thus till the last day. Cells exposed to pGLu883ΔXba formed a lighter tissue that started to show CPE at the 5th day of incubation, the 8th day the cells presented 100% CPE. The same results were observed for the cells electroporated with dspBLu(+). In the flasks with sspBLu(+) uncut and pBLu(+)/Dpn I no CPE was observed after the incubation period was completed, meaning that in fact no ssDNA was present.

Table 10. **Electroporation of *HeLa* cells using ssDNA generated using a helper phage**

25cm^3 Flask	**DNA**	**Observations after 8 days**
Control	None	Tissue layer
Positive control	pGLu883ΔXba	100% CPE
ds(+) uncut	pBLu(+)	100% CPE
ss(+) uncut	sspBLu(+) uncut	Tissue layer
ss(+)/Dpn I	sspBLu(+) digested with Dpn I	Tissue layer

The attempt to produce ssDNA using a helper phage did not yield positive results. The protocol was repeated several times without success. The next step was trying to generate the ssDNA using the Polymerase chain reaction (PCR).

II. Single-stranded DNA generated by PCR. The template used was pBLu(+) digested with the enzyme *Pvu I*. In theory submitting the template to amplification with excess of a single primer would produce one of the strands, the polarity of the strand would depend on the primers used. The Forward primer would produce the (-) strand, whereas the Reverse primer would produce the (+) strand. No product was expected from the reaction without primers, and the reaction with both primers was used as a positive control that would yield a double-stranded product. For the four individual PCR reactions the same product was obtained, a fragment of ~5 Kb that corresponds to dsDNA, including the one deprived of primers. This result can be attributed to the capability of the terminals of the LuIII genome to fold on themselves and form hairpins that can act as primers for the PCR reactions. These results suggest that the PCR method is a very unlikely procedure to obtain ssDNA of LuIII.

III. Single-Stranded DNA generated by Exonuclease III treatment. The enzyme *Exonuclease III* (Exo III) has a double-strand specific, nonprocessive 3' ⟶ 5' exodeoxyribonuclease activity; however 3'-overhangs of ≥4 bases are protected from Exo III activity; this means, that the enzyme catalyzes the stepwise removal of mononucleotides starting from 3'-OH at: 1) blunt ends, 2) recessed ends, and 3) nicks. It will also act on 3'-overhangs of less than 4 bases.

For this assay two sets of enzymes were used for double digestions. On the first set of enzymes, *Eco RV* (blunt) and *Sac I* (recessed: 3'-overhang of 4 bases), Exo III removes mononucleotides starting at the 3'-0H of the blunt end (*Eco RV*), located on the (-) strand, and yielding the (+) strand. For the second set, *Pst I* (recessed: 3'-overhang of 4 bases) and *Spe I* (blunt), the removal of mononucleotides occurs on the (+) strand, leaving only the (-) strand (Figure 13, Chapter III).

After treatment of the double digestions with Exo III, another digestion, this time with *Dpn I,* was performed, to eliminate any double-stranded DNA left behind. All the samples were resolved in a 1% agarose gel in TAE 1X buffer at 65V for 4 hours. A difference in migration can be observed in the samples treated with Exo III versus the ones that weren't. The fragments of our interest a ~5 kb that can be seen in both double digestions, is expected to run at about 3 kb in the lanes that correspond to the Exo III treated samples. In Figure 20 the migration of the DNA as is being treated with Exo III can be appreciated, the reaction was stopped at different time points. After digestion with *Dpn I* this fragment remained, suggesting that the fragment corresponds to ssLuIII DNA. There is no difference in the migration of the (+) and the (-) strands.

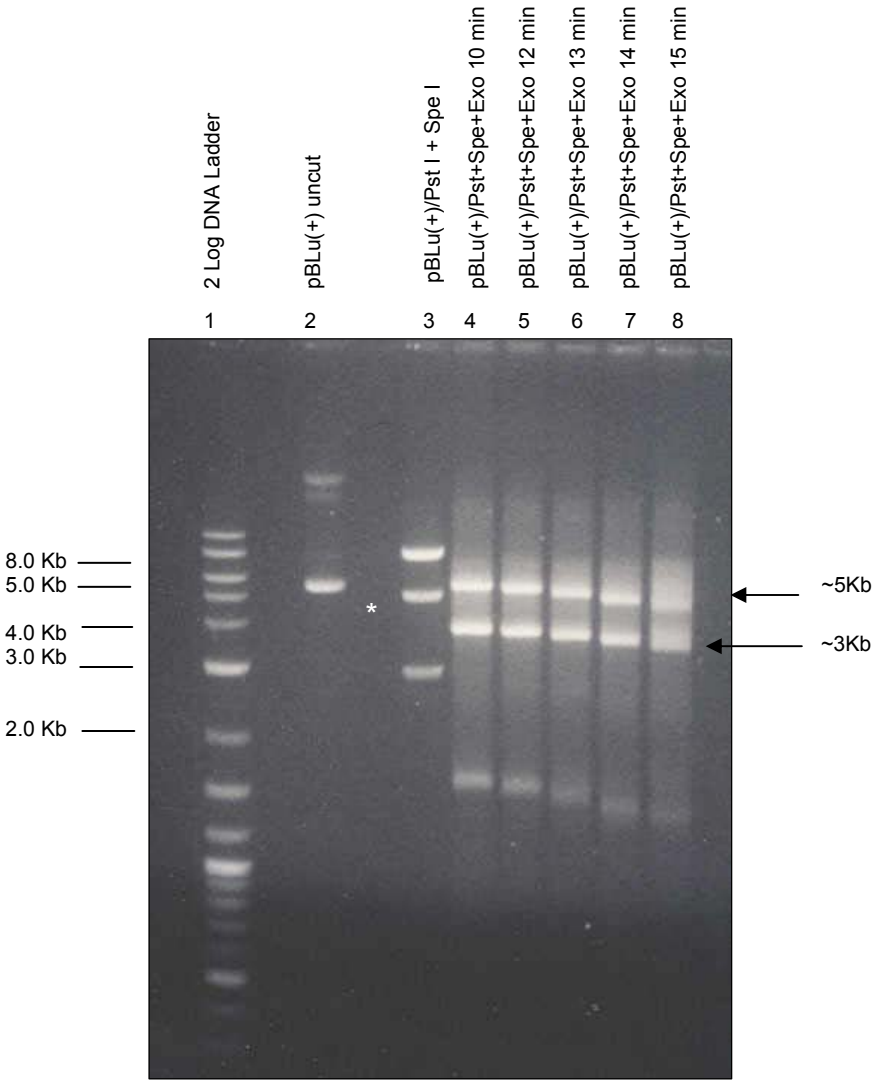


Figure 20. **Time assay of pBLu(+) treated with *Exonuclease III.***

* Fragment of ~5kb that corresponds to the LuIII genome

Bacterial transformation. Results of the transformation of these samples into XL1-Blue cells are depicted in Table 11 below; this data suggests that the digestions and Exo III treatment were successful in linearizing and modifying the DNA since no circular (uncut) plasmid was recovered.

Table 11. **Transformation of bacterial cells with LuIII ssDNA generated with *Exonuclease III*.**

Tube	DNA	CFU/µL
Blank w/o Amp Blank w/Amp	None	Clear Bacterial lawn
Standard	pBluescript II SK(+)	400
ds(+)	pBLu(+) uncut	250
(+)/E+S	pBLu(+) cut with *Eco RV* and *Sac I*	0
(+)/P+S	pBLu(+) cut with *Pst I* and *Spe I*	1
(+)/E+S+E	pBLu(+) cut with E+S and treated with Exo III	0
(+)/P+S+E	pBLu(+) cut with P+S and treated with Exo III	0
(+)/E+S+E+D	ssBLu(+) digested with *Dpn I*	0
(+)/P+S+E+D	ssBLu(+) digested with *Dpn I*	0
(+)/E+S+E+D P+S+E+D	sspBLu(+) of both polarities	0

Transfection of HeLa cells using DNA obtained from Exonuclease III treatment. Transfected cells were maintained and observed for 12 days at 37°C. No significant changes were observed during the first 5 days post transfection, cells looked healthy and confluent. On the 6th day the first CPE signs began to appear. Table 12 summarizes the results of the microscopic

observations, and in Figure 20 we can see how de cells looked at days 2, 6 and 10 after transfection.

In the cell control flask (no DNA added), the cells looked confluent and healthy until the 10th day after transfection, when they started to look stressed because of the high confluence, space, and medium constraints. The cells were recovered from the electroporation procedure, therefore any cell lysis that might appear during the days to fallow in the positive controls and experimentals is likely due to viral infection. In the flask transfected with pGLu883ΔXba (positive control), cells looked healthy and confluent until the 6th day post-transfection, when they started to show a localized infection, eventually reaching 100% CPE on the 10th day post transfection. A similar infection pattern was observed for cells transfected with pBLu(+) uncut, which confirms the capacity of the pBLu(+) construct to cause infection, just as pGLu883ΔXba. Another flask of cells was transfected with the construct pBLu(+) cut with the enzyme *Bam HI*, which liberates the LuIII genome from the vector pUC19; this linear molecule best resembles the ss LuIII DNA that we are using to transfect the experimental flasks. No difference was observed between the cells in this flask and the cells in the flasks transfected with pGLu883ΔXba and pBLu(+). Infection in the three flasks proceeded in a similar manner, reaching 100% CPE in 10 days.

For the flasks transfected with samples that were not submitted to *Dpn I* digestion, pBLu(+)/E+S+E and pBLu(+)/P+S+E, which likely have a mixture of both dsDNA and ssDNA, the first CPE signs did not appear until the 7th day post transfection, and the infection progressed very slowly. By the 10th day, when the positive controls had reached 100% CPE, these cells only presented 60% CPE, reaching 100% cell lysis by the 15th day. This phenomenon may be explained by a competition for the replication machinery between the dsDNA and the ssDNA, resulting in a slower replication rate and viral infection.

The cells transfected with the (-) strand (pBLu(+)/P+S+E+D) and with the (+) strand (pBLu(+)/E+S+E+D) independently presented CPE. For the (-) strand the infection occurred more synchronized through out the flask, just as the double-stranded constructs, reaching 100% CPE on the 10th day post-tranfection. For the (+) strand the infection appeared more localized and 100% CPE was reached on the 12th day after transfection. Infection in these flasks was quicker than in the ones that were not treated with *Dpn I*, which suggests that the ss viral DNA alone is more effective than the mixture of ss and ds DNA; no difference was observed between the cells transfected with pGLuΔXba (positive control) and the ones transfected with the ss LuIII (-) strand, both infections progressed similarly. There is a notable difference between the effectiveness of the (-) strand over the (+) strand in causing CPE, this delay may be explained by the fact that the (+) strand will require a (-) strand intermediate for synthesis of viral mRNAs, whereas the (-) strand could serve directly as a template for mRNAs transcription.

Table 12. **Daily observations of HeLa cells transfected to assess infectivity capacity of the samples**

DAY OF TRANSFECTION (HOURS)	CONTROLS				EXPERIMENTALS				
	Cells	**pGLu883ΔXba**	**pBLu(+) uncut**	**pBLu(+) Bam HI**	**pBLu(+) E+S+E**	**pBLu(+) E+S+E+D**	**pBLu(+) P+S+E**	**pBLu(+) P+S+E+D**	**(+)+(-) strands Combined**
6 (144 hours)	Healthy, very confluent	Infected 25% CPE	Infected 25% CPE	Infected 25% CPE	Healthy, extremely confluent	Infected 10% CPE	Healthy, extremely confluent	Infected 25% CPE	Infected 25% CPE
7 (168 hours)	Healthy, very confluent	Infected 50% CPE	Infected 50% CPE	Infected 50% CPE	Infected 10% CPE	Infected 25% CPE	Infected 10% CPE	Infected 50% CPE	Infected 50% CPE
8 (192 hours)	Healthy, very confluent	Infected 80% CPE	Infected 80% CPE	Infected 80% CPE	Infected 30% CPE	Infected 40% CPE	Infected 30% CPE	Infected 80% CPE	Infected 80% CPE
9 (216 hours)	Healthy, extremely confluent	Infected 90% CPE	Infected 90% CPE	Infected 90% CPE	Infected 40% CPE	Infected 50% CPE	Infected 40% CPE	Infected 90% CPE	Infected 90% CPE
10 (240 hours)	Healthy, extremely confluent	Infected 100% CPE	Infected 100% CPE	Infected 100% CPE	Infected 60% CPE	Infected 70% CPE	Infected 60% CPE	Infected 100% CPE	Infected 100% CPE
11 (264 hours)	Healthy, extremely confluent				Infected 70% CPE	Infected 90% CPE	Infected 70% CPE		
12 (288 hours)	Healthy, extremely confluent				Infected 80% CPE	Infected 100% CPE	Infected 80% CPE		

Note: These CPE results represent qualitative microscopic observations.

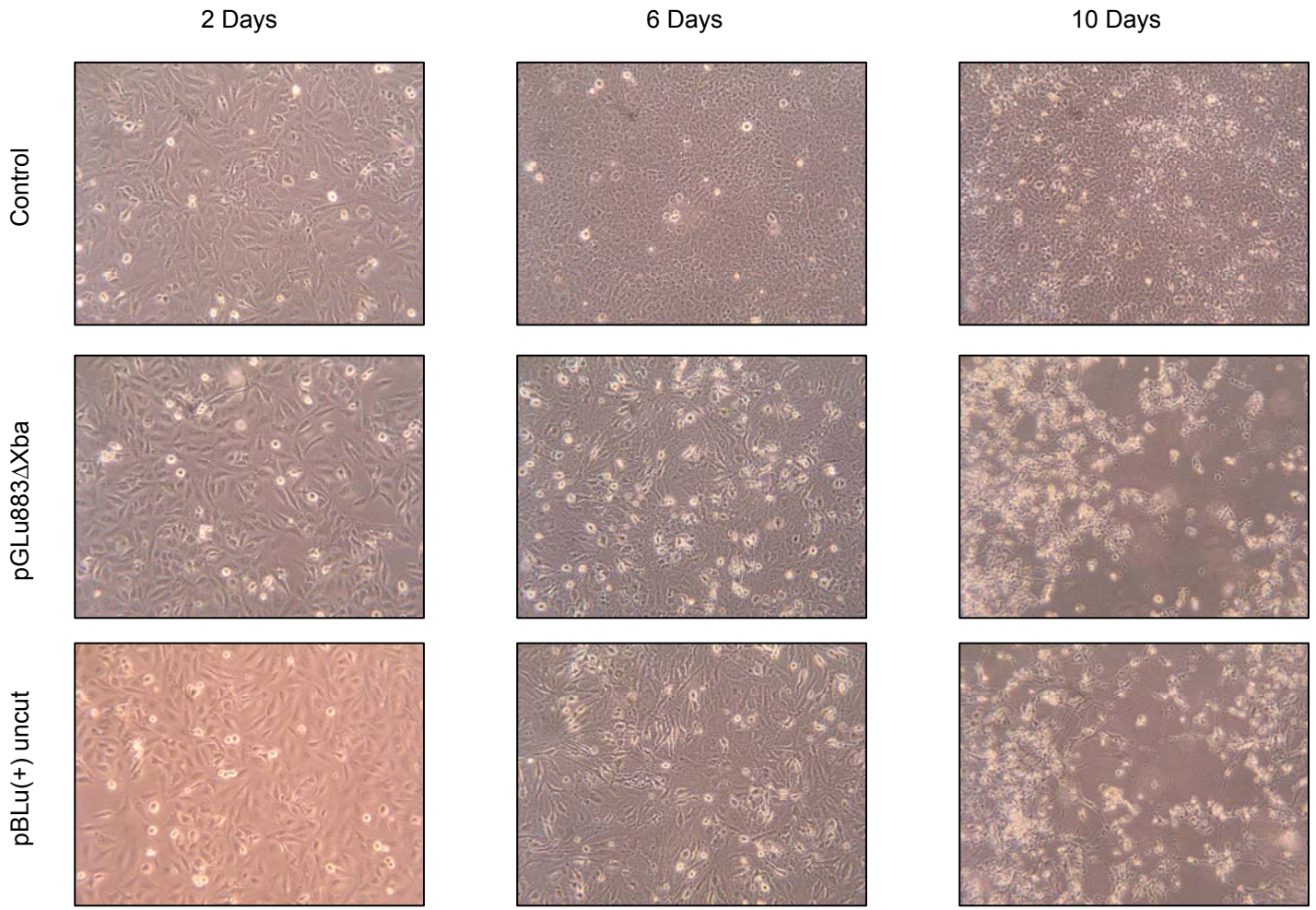
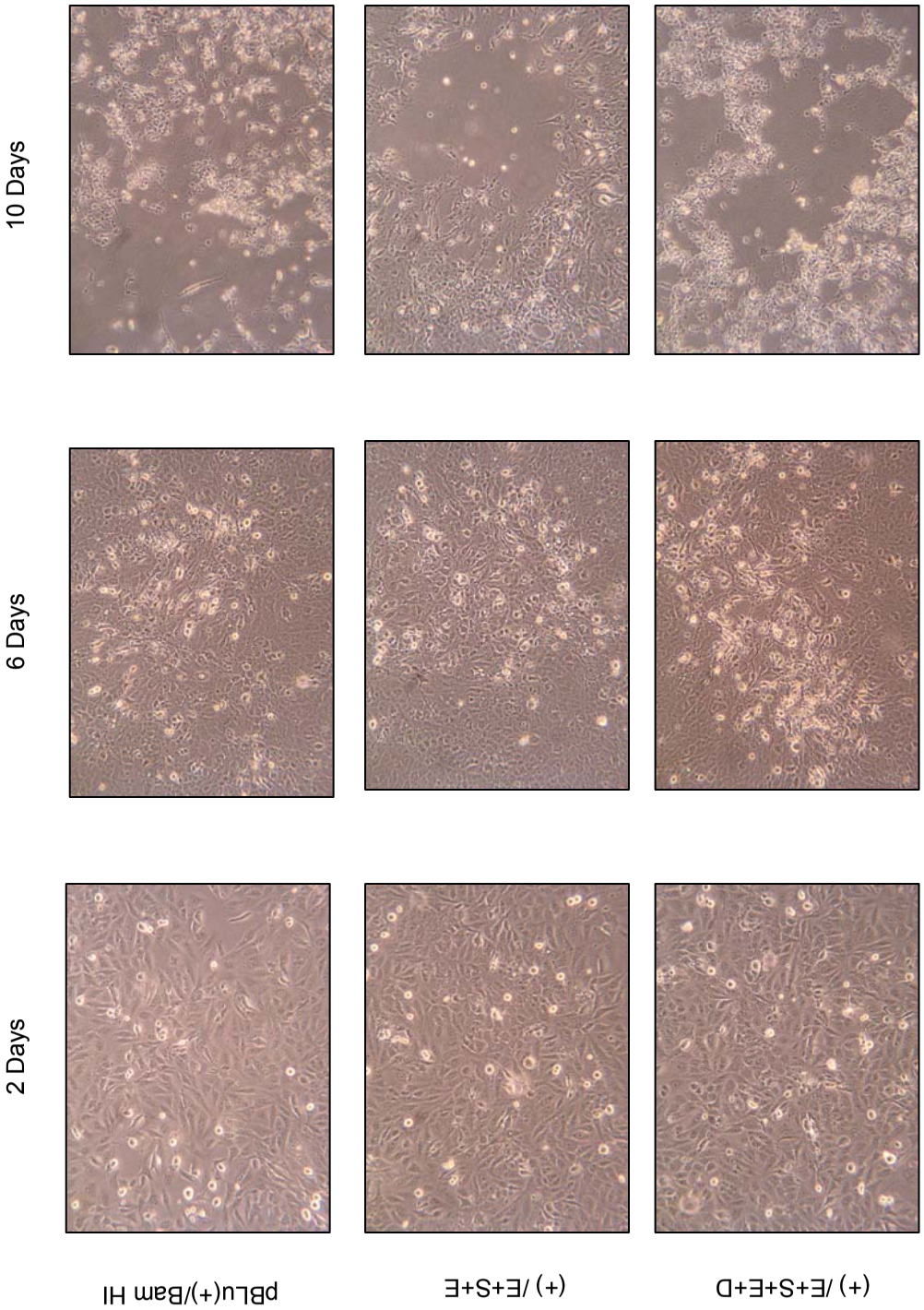


Figure 21. **HeLa cells electroporated with samples from Exo III treatment.**


10 Days
6 Days
2 Days
pBLu(+)/Bam HI
(+) /E+S+E
(+) /E+S+E+D

2 Days
6 Days
10 Days
(+) /P+S+E
(+) /P+S+E+P
(+) /E+S+E+D
P+S+E+D

Rescue of viral particles from transfected cells and infection of HeLa cells. After cells reached 100% CPE, viral particles were extracted from each flask and viral stocks were made. These stocks were used to infect HeLa cells and confirm that indeed full viral particles capable of establishing an infection were produced. Cell lysis occurred 5 days after the infection on all samples, and no difference could be observed between the samples.

CHAPTER V
CONCLUSIONS

- Two clones were constructed using the vector pBluescript II SK(+) and the LuIII genome: one with the LuIII fragment inserted in the 0-100 m.u. direction, pBLu(+), and the other one with the genome inserted in the 100-0 m.u. direction, pBLu(-).

- Three different protocols were performed to obtain ssDNA from this clones. The first one using a helper phage, and the second using PCR were unsuccesful. The third one, using *Exonuclease III,* yielded the desired (+) and (-) strands from the pBLu(+) construct.

- The ss(+) and ss(-) strands were transfected into cancerous HeLa cells, both strands proved to be capable of establishing a productive infection.

- The (-) strand appeared to be more effective than the (+) strand in establishing the viral infection, it showed a more widespread infection and reached complete cell lysis 2 days prior to the (+) strand.

- Cotransfection with ss viral DNA and ds viral DNA appears to retard the replication process and hence the establishment of the infection, regardless of the polarity of the strand used for the cotransfection.

- Particles obtained from each flask were able to infect HeLa cells reaching 100% CPE after 5 days of infection.

- Cotransfection with the ss(+) and ss(-) LuIII strands behaved similarly to transfection with the infectious clone pBLu(+).

CHAPTER VI
RECOMMENDATIONS

The following recommendations should be considered :

- Repeat the process, from the generation of the ss viral DNA to the transfection of HeLa cells, to confirm the results previously obtained.

- Tranfect HeLa cells with the ss LuIII viral DNA and perform a time assay to observe if transfection with the independent strands results in similar replication intermediates.

- Extract DNA from cells transfected with ss LuIII DNA, and confirm the polarity of the strands produced after transfection with the ss(-) strand and with the ss(+) strand.

BIBLIOGRAPHY

1. Adbandje-McKenna, M., A. Llamas-Saiz, F. Wang, P. Tattersall, and M. Rossmann. 1998. Functional implications of the structure of the murine parvovirus, minute virus of mice. Structure 6(11): 1369-1381.

2. Ali, M., N.R. Lemoine, and C.J.A. Ring. 1995. The use of DNA viruses as vectors for gene therapy. Gene Therapy 1(6): 367-384.

3. Astell, C.R., M. Smith, M.B. Chow, and D.C. Ward. 1979. Structure of the 3' hairpin termini of four rodent parvovirus genomes: nucleotide sequence homology at origins of DNA replication. Cell 17(3):691-703.

4. Astell, C.R., M. Thomson, M. Merchlinsky, and D.C. Ward. 1983. The complete nucleotide sequence of Minute Virus of Mice, an autonomous parvovirus. Nucleotide Acid Research 11 (4): 999-1018.

5. Ausubel, F.M., B. Roger, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1994. Short Protocols in Molecular Biology 3rd Ed. Current Protocols, Wiley, USA.

6. Avalosse, B., F. Dupont, P. Spegelaere, N. Mine, and A. Burny. 1997. Method for concentrating and purifying recombinant autonomous parvovirus vectors designed for tumor-cell-targeted gene therapy. Journal of Virological Methods 62: 179-183.

7. Baldauf, A.Q., K. Willwand, E. Mumtsidu, J.P. Nüesch, and J. Rommelaere. 1997. Specific initiation of replication at the right-end telomere of the closed species of minute virus of mice replicative-form DNA. Journal of Virology 71(2): 971-980.

8. Bando, H., H. Choi, Y. Ito, and S. Kawase. 1990. Terminal structure of a densovirus implies a hairpin transfer replication which is similar to the model for AAV. Virology 179: 57-63.

9. Bashir, T., R. Hörlein, J. rommelaere, and K. Willwand. 2000. Cyclin A activates the DNA polymerase δ-dependent elongation machinery *in vitro*: a parvovirus DNA replication model. Proceedings of the National Academy of Sciences USA 97(10): 5522-5527.

10. Bates, R.C., C.E. Snyder, P.T. Banerjee, and S. Mitra. 1984. Autonomous parvovirus LuIII encapsidates equal amounts of plus and minus DNA strands. Journal of Virology 49(2): 319-324.

11. Bergoin, M., and P. Tijssen. 1998. Biological and molecular properties of densoviruses and their use in protein expression and biological control. The Insect Viruses. The Virus. Plenum Press, N.Y., pp 141-169.

12. Berns, K.I. 1996. Parvoviridae: The Viruses and their Replication. In: B.N. Fields, and D.M. Knipe (ed.) Fundamental Virology, pp 1017-1041.

13. Brown, C., F. DiSumma, J. Rommelaere, A.Y. Dege, J. Cornelis, C. Dinsart, and W.J. Spaan. 2002. Production of recombinant H-1 parvovirus stocks devoid of replication-competent viruses. Human Gene Therapy 13(18): 2135-2145.

14. Chen, K. C., B. C. Shull, M. Lederman, E. R. Stout, and R. C. Bates. 1988. Analysis of the termini of the DNA of Bovine Parvovirus: demonstration of sequence inversion at the left terminus and its implication for the replication model. Journal of Virology. 62: 3807-3813.

15. Christensen, J., S. F. Cotmore, and P. Tattersall. 1995. Minute virus of mice transcriptional activator protein NS1 binds directly to the transactivation region of the viral P38 promoter in a strictly ATP-dependent manner. Journal of Virology 69(9): 5422-5430.

16. Christensen J., S. F. Cotmore, and P. Tattersall. 1997. A novel cellular site-specific DNA-binding protein cooperates with the viral NS1 polypeptide to initiate parvovirus DNA replication. Journal of Virology 71: 1405-1416.

17. Christensen, J., and P. Tattersall. 2002. Parvovirus initiator protein NS1 and RPA coordinate replication fork progression in a reconstituted DNA replication system. Journal of Virology 76: 6518-6531.

18. Corsini, J., J. O. Carlson, F. Maxwell, and I. Maxwell. 1995. Symmetric-strand packaging of recombinant parvovirus LuIII genomes that retain only the terminal regions. Journal of Virology 69(4): 2692-2696.

19. Corsini, J., S.F. Cotmore, P. Tattersall, and E. Winocours. 2001. The left-end and right-end origins of minute virus of mice DNA differ in their capacity to direct episomal amplification and integration *in vivo*. Virology 288: 154-163.

20. Cossons, N., E. Faust, and M. Zannis-Hadjopoulos. 1996. DNA polymerase δ-dependent formation of a hairpin structure at the 5' terminal palindrome of the minute virus of mice genome. Virology 216: 258-264.

21. Cotmore, S. and P. Tattersall. 1986. Organization of nonstructural genes of the autonomous parvovirus minute virus of mice. Journal of Virology 58(3): 724-732.

22. Cotmore, S. and P. Tattersall. 1988. The NS1 polypeptide of minute virus of mice is covalently attached to the 5' termini of duplex replicative-form DNA and progeny single strands. Journal of Virology 62(3): 851-860.

23. Cotmore, S., A.M. D'Abramo Jr., L.F. Carbonell, J. Bratton, and P. Tattersall. 1997. The NS2 polypeptide of parvovirus MVM is required for capsid assembly in murine cells. Virology 231, 267-280.

24. Deleu, L., F. Fucks, D. Spitkovsky, R. Hörlein, S. Faisst, and J. Rommelaere. 1998. Opposite transcriptional effects of cyclic AMP-responsive elements in confluent or $p27^{kip}$-overexpressing cells versus serum-starved or growing cells. Molecular and Cellular Biology 18(1): 409-419.

25. Diffoot N., B.C. Shull, K.C. Chen, E.R. Stout, M. Lederman, and R. Bates. 1989. Identical ends are not required for the equal encapsidation of plus- and minus-strand parvovirus LuIII DNA. Journal of Virology 63(7): 3180-3184.

26. Diffoot, N., K.C. Chen, R.C. Bates, and M. Lederman. 1993. The complete nucleotide sequence of parvovirus LuIII and localization of a unique sequence possibly responsible for its encapsidation pattern. Virology 192: 339-345.

27. Di Pasquale, G., and S.N. Stacey. 1998. Adeno-associated virus Rep78 protein interacts with protein Kinase A and its homolog PRKX and inhibits CREB-dependent transcriptional activation. Journal of Virology 72(10): 7916-7925.

28. Dumas, B., M. Jourdan, A.M. Pascaud, and M. Bergoin. 1992. Complete nucleotide sequence of the cloned infectious genome of *Junonia coenia* densovirus reveals an organization unique among parvoviruses. Virology 191: 202-221.

29. Dupont, F., L. Tenenbaum, L.P. Guo, P. Spegelaere, M. Zeicher, and J. Rommelaere. 1994. Use of an autonomous parvovirus vector for selective transfer of a foreign gene into transformed human cells of different tissue origins and its expression therein. Journal of Virology 68(3): 1397-1406.

30. Dupont., F., B. Avalosse, A. Karim, N. Mine, M. Bosseler, A. Maron, A.V. Van den Broeke, G.E. Ghanem, A. Burny, and M. Zeicher. 2000. Tumor-selective gene transduction and cell killing with an oncotropic autonomous parvovirus-based vector. Gene Therapy 7(9): 790-796.

31. Eichwald, V., L. Daeffler, M. Klein, J. Rommelaere, and N. Salomé. 2002. The NS2 protein of parvovirus minute virus of mice are required for efficient nuclear egress of progeny virions in mouse cells. Journal of Virology 76(20): 10307-10319.

32. Faisst, S.; D. Guittard, A. Benner, J.Y. Cesbron, J.R. Schlehofer, J. Rommelaere, and T. Dupressoir. 1998. Dose-dependent regression of Hela-cell derived tumors in scid mice after parvovirus H-1 infection. International Journal of Cancer 75: 584-589.

33. Faisst, S., and J. Rommelaere (Eds). 2000. Parvoviruses: From Molecular Biology to Pathology and Therapeutic uses. Contrib. Microbiol. Basel, Karger, Vol. 4.

34. Fernandez, J., H. Muller, M. Ruffing, and J.A. Kleinschmidt. 1995. Influence of AAV-2 terminal repeats on expression of heterologous promoters. Biotecnologia Aplicada 12(1): 52-56.

35. Gancberg, D., M. Zeicher, M. Bakkus, F. Dupont, O. Leo, M. Moser, P. Spegelaere, K. Thielemans, J. Urbain, and M. Horth. 2000. Oncoselective transduction of CD80 and CD86 in tumor cell lines using an autonomous recombinant parvovirus. Anticancer Research 20(3A): 1825-1832.

36. Gareus, R.; A. Gigler, A. Hemauer, M. Leruez-Ville, F. Morinet, H. Wolf, and S. Modrow. 1998. Characterization of *cis*-acting and NS1 protein-responsive elements in the p6 promoter of parvovirus B19. Journal of Virology 72(1): 609-616.

37. Gersappe, A.; L. Burger, and D.J. Pintel. 1999. A premature termination codon in either exon of minute virus of mice p4 promoter-generated pre-mRNA can inhibit nuclear splicing of the intervening intron in an open reading frame-dependent manner. Journal of Biological Chemistry 274(32): 22452-22458.

38. Giraud, C.; G. Devauchelle, and M. Bergoin. 1992. The densovirus of *Junonia coenia* (Jc DNV) as an insect cell expression vector. Virology 186: 207-218.

39. Haag, A., P. Menten, J. Van Damme, C. Dinsart, J. Rommelaere, and J.J. Cornelis. 2000. Highly efficient transduction and expression of cytokine genes in human tumor cells by means of autonomous parvovirus vectors; generation of antitumor responses in recipient mice. Human Gene Therapy 11(4): 597-609.

40. Heegard, E., and A. Hornsleth. 1995. Parvovirus: The expanding spectrum of disease. Acta Paediatr. 84: 109-117.

41. Hermonat, P.L. 1994. Down-regualtion of human c-*fos* and c-*myc* proto-oncogene promoters by adeno-associated virus Rep78. Cancer Letters 81(2): 129-136.

42. Kessler P. D. et al. 1996. Gene delivery to skeletal muscle results in sustained expression and systematic delivery of a therapeutic protein. Proceedings of the National Academy of Sciences USA 93:14082-14087.

43. Kestler, J., B. Neeb, S. Struyf, J. Van Damme, S.F. Cotmore, A. D'Abramo, P. Tattersall, J. Rommelaere, C. Dinsart, and J.J. Cornelis. 1999. *cis* Requirements for the efficient production of recombinant DNA vectors based on autonomous parvoviruses. Human Gene Therapy 10: 1619-1632.

44. Krady, J.K., and D.C. Ward. 1995. Transcriptional activation by the parvoviral nonstructural protein NS1 is mediated via a direct interaction with Sp1. Molecular and Cellular Biology 15(1): 524-533.

45. Kuntz-Simon, G., T. Bashir, J. Rommelaere, and K. Willwand. 1999. Neoplastic transformation-associated stimulation of the in vitro resolution of concatemer junction fragments from Minute Virus of Mice DNA. Journal of Virology 73: 2552-2558.

46. Lopez-Guerrero, J.A.; B. Rayet, M. Tuynder, J. Rommelaere, and C. Dinsart. 1997. Constitutive activation of U937 promonocyte cell clones selected for their resistance to parvovirus H-1 infection. Blood 89(5): 1642-1653.

47. Lorson, C.; L.R. Burger, M. Mouw, and D.J. Pintel. 1996. Efficient transactivation of the minute virus of mice p38 promoter requires upstream binding of NS1. Journal of Virology 70(2): 834-842.

48. Lukashov, V., and J. Goudsmit. 2001. Evolutionary relationships among parvoviruses: virus-host coevolution among autonomous primate parvoviruses and links between adeno-associated and avian viruses. Journal of Virology 75(6): 2729-2740.

49. Maxwell, I., F. Maxwell, S.L. Rhode, J. Corsini and J.O. Carlson. 1993. Recombinant LuIII autonomous parvovirus as a transient transducing vector for human cells. Human Gene Therapy 4: 441-450.

50. Maxwell, I., A. Spitzer, C.J. Long, and F. Maxwell. 1996. Autonomous parvovirus transduction of a gene under control of tissue-specific or inducible promoters. Gene Therapy 3(1): 28-36.

51. Monahan, P. E., and R. J. Samulski. 2000. AAV vectors: is clinical success on the horizon? Gene Therapy 7: 24-30.

52. Mousset, S., Y. Quadrhiri, P. Caillet-Fauquet, and J. Rommelaere. 1994. The cytotoxicity of the autonomous parvovirus minute virus of mice nonstructural proteins in FR3T3 rat cell depends on oncogene expression. Journal of Virology 68(10): 6446-6453.

53. Nüesch, J.P.F., S. Dettwiler, R. Corbau, and J. Rommelaere. 1998. Replicative functions of minute virus of mice NS1 protein are regulated *in vitro* by phosphorylation through protein kinase C. Journal of Virology 72(12): 9966-9977.

54. Nüesch, J.P.F., S. Lachmann, R. Corbau, and J. Rommelaere. 2003. Regulation of minute virus of mice replicative functions by atypical PCKλ *in vivo.* Journal of Virology 77(1): 433-442.

55. Op De Beeck, A.; F. Anouja, S. Mousset, J. Rommelaere and P. Caillet-Fauquet. 1995. The nonstructural proteins of the autonomous parvovirus minute virus of mice interfere with the cell cycle, inducing accumulation in G-2. Cell Growth and Differentiation 6(7): 781-787.

56. Op De Beeck, A., and P. Caillet-Fauquet. 1997. The NS1 protein of the autonomous parvovirus minute virus of mice blocks cellular DNA replication: a consequence of lesions to the chromatin? Journal of Virology 71(7): 5323-5329.

57. Pallier, C.; A. Greco, J. Le Junter, A. Saib, I. Vassias, and F. Morinet. 1997. The 3' untranslated region of the B19 parvovirus capsid protein mRNAs inhibits its own mRNA translation in nonpermissive cells. Journal of Virology 71(12): 9482-9489.

58. Pintel, D., D. Dadachanji, C.R. Astell and D.C. Ward. 1983. The genome of minute virus of mice, an autonomous parvovirus encodes two overlapping transcriptional units. Nucleic Acid Research 11(4): 1019-1038.

59. Renuka, K-M., and J.P. Trempe. 1995. The adeno-associated virus Rep78 protein is covalently linked to viral DNA in a preformed virion. Virology 214: 360-370.

60. Rolling, F., and R.J. Samulski. 1995. AAV as a viral vector for human gene therapy: Generation of a recombinant virus. Molecular Biotechnology 3(1): 9-15.

61. Spegelaere, P., J.J. Cornelis, M. Tuynder, and J. Rommelaere. 1994. Lack of detectable effect of capsid proteins on the cell-dependent activity of parvovirus MVMp promoters. Research in Virology 145, 5-12.

62. Spitzer, A.L., C.R. Parrish, and I.H. Maxwell. 1997. Tropic determinant for canine parvovirus and feline panleukopenia virus functions through the capsid protein VP2. Journal of General Virology 78(4):925-928.

63. Tattersall, P. and S.F. Cotmore. 1988. The nature of Parvoviruses. In J.R. Pattison (ed.) Parvoviruses and Human Diseases. Pp 6-37. CRC Press, Boca Raton, FL, USA.

64. Tullis, G.; R.V. Schoborg, and D.J. Pintel. 1994. Characterization of the temporal accumulation of minute virus of mice replication intermediates. Journal of General Virology 75(7): 1633-1646.

65. Vincent, K.A.; S.T. Piraino, and S.C. Wadsworth. 1997. Analysis of recombinant adeno-associated virus packaging and requirements for *rep* and *cap* gene products. Journal of Virology 71(3): 1897-1905.

66. Weichert, W.S., J.L.S. Parker, A.T.M. Wahid, S.F. Chang, E. Meier, and C. Parrish. 1998. Assaying for structural variation in the parvovirus capsid and its role in infection. Virology 250:106-117.

67. Willwand, K., E. Mumtsidu, G Kuntz-Simon, and J. Rommelaere. 1998. Initiation of DNA replication at palindromic telomeres is mediated by a duplex-to-hairpin transition induced by the Minute Virus of Mice nonstructural protein NS1. Journal of Biological Chemistry 273: 1165-1174.

68. Woolf, A.D.; G.V. Campion, A. Chishick, S. Wise, and B.J. Cohen. 1989. Manifestations of human parvovirus B19 in adults. Archives of Internal Medicine 149: 153-156.

69. Xiao X., J. Li, and R. Samulski. 1996. Efficient long term transfer into muscle tissue of immunocompetent mice by Adeno-associated virus vector. Journal of Virology 70: 8098-8108.

70. Yang, C.C.; X. Xiao, X. Zhu, D.C. Ansardi, N.D. Epstein, M.R. Frey, A.G. Matera, and R.J. Samulski. 1997. Cellular recombination pathways and viral terminal repeat hairpin structures are sufficient for adeno-associated virus integration *in vivo* and *in vitro*. Journal of Virology 71(12): 9231-9247.