REPLICATION OF THE 3' LUIII 3' SUBGENOMIC CLONE IN HUMAN CELLS

By

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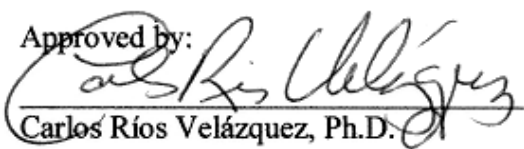
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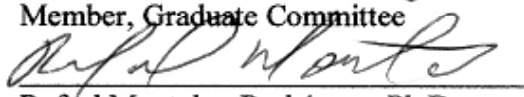in
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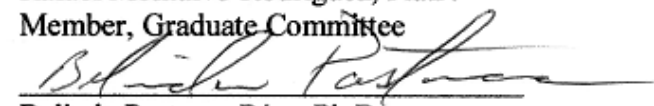Carlos Ríos Velázquez, Ph.D.   Date
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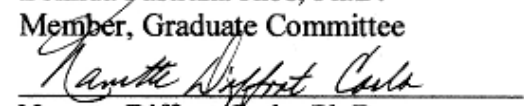Member, Graduate Committee

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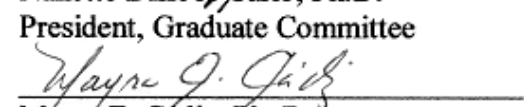Member, Graduate Committee

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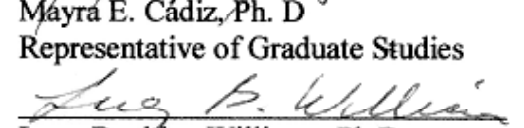President, Graduate Committee

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Abstract

Parvoviruses are small, non-enveloped, icosahedral viruses that contain a linear, single-stranded DNA genome of approximately 5000 bp. Parvovirus LuIII is an autonomous parvovirus that shares up to 80% of sequence homology with the rodent parvovirus MVM and H1, but encapsidates equal amounts of plus and minus strand, identical to AAV and B19. To study the possibility that the termini determine the strand to be encapsidated, a plasmid containing two copies of the left end termini was initially constructed. However, this plasmid known as 3' LuIII 3' has an insertion of unknown origin between the two termini. To eliminate this stuffer sequence, three different strategies were followed. The first two strategies consisted in eliminating the stuffer sequence by digesting the plasmid independently with *Pme*I and *Mlu*I enzymes, and then re-circulating it, and the third strategy consisted in constructing the recombinant molecule *de novo*. Irrespective of the strategy used, the desired 3' LuIII 3' clone was not obtained. To determine if the original clone 3' LuIII 3', was able of replicating in eukaryotic cells, the plasmid was co-transfected into HeLa cells with the infectious clone pGLu883Δ*Xba*. No evidence of 3' LuIII 3' replication was observed.

Resumen

Los parvovirus son pequeños virus icosaédricos sin envoltura que contienen un DNA lineal de hebra sencilla de aproximadamente 5000 pb. El virus LuIII es un parvovirus autónomo que comparte hasta un 80% de homología con los parvovirus autónomos MVM y H-1, pero éste encapsida cantidades iguales de hebras positiva y negativa. Este patrón es idéntico a AAV y B-19. Para estudiar la posibilidad de que los terminales estén determinando la polaridad de la hebra que se encapsida, un plásmido que contiene dos copias del terminal 3' fue construido. Sin embargo, este plásmido llamado 3' LuIII 3', tiene una inserción de origen desconocido entre los dos terminales. Tres estrategias distintas se llevaron a cabo para eliminar esta secuencia. Las primeras dos estrategias consistieron en eliminar esta secuencia desconocida cortando el plásmido por separado con las enzimas *Pme*I y *Mlu*I. La tercera estrategia consistió en construir la molécula recombinante *de novo*. Independientemente de la estrategia utilizada, no se obtuvo un clon 3' LuIII 3' como fuera deseado. Para determinar si el clon original 3' LuIII 3' era capaz de replicarse en células eucarióticas, el plásmido fue co-transfectado con el clon infeccioso pGLu883Δ*Xba* en células HeLa, no observándose evidencia de replicación para el plásmido 3' LuIII 3'.

Dedication

I dedicate this work to my cherished mother for being my inspiration and my angel, to my dearest father for his unconditional care and support, and to you Carlos for giving me the greatest gift, your love.

Acknowledgements

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CHAPTER I
Introduction

Autonomous parvoviruses (APVs) are small, non-enveloped, icosahedral viruses that are currently being studied for human gene therapy. They can only replicate in the nucleus during the S period of the host cell cycle. The viral DNA has short palindromic terminal sequences capable of folding into hairpin duplexes. The left hairpin serves as template and primer for complementary-strand synthesis to produce a double stranded replicative form, which closely resembles the rolling-circle DNA replication mechanism, previously characterized in prokaryotic replicons.

The single-stranded DNA encapsidated by parvoviruses can be of plus or minus polarity and the ratio varies among members. Most autonomous parvoviruses have different 3' and 5' terminal sequences and encapsidate primarily minus strand. The autonomous parvovirus LuIII encapsidates both DNA strands with equal frequency, yet, its terminal sequences share over 90% homology with the termini of the rodent parvoviruses which encapsidate 99% minus strand. The palindromic terminal sequences of parvoviruses are essential for replication. The replication models proposed for autonomous parvoviruses suggest that the left end hairpin acts as an origin of replication. The consequences of the presence of two left ends in replication and/or the encapsidation pattern are unknown.

In order to study the role of the left terminus in the replication and encapsidation pattern of the autonomous parvovirus LuIII, a minigenome containing two copies of the left end palindrome (nt 1-277) cloned into the *Eco* RI site of pUC19 was constructed. This construct, named 3' LuIII 3', contains an insertion of unknown sequence between the two termini. This unknown stuffer sequence will be eliminated before it is transfected into human cells, and its encapsidation pattern compared to that of the wild type genome.

The possible use of LuIII as a transient genetic vector for therapeutic purposes can only occur if the basic biological functions are fully understood. This work attempts to understand the mechanism used by the virus LuIII to replicate its genome and encapsidates both strands with similar frequency.

CHAPTER II
Literature Review

Parvoviridae

The *Parvoviridae* family includes a broad variety of viruses that replicate in the nuclei of both invertebrate and vertebrate hosts. They are small, non-enveloped, icosahedral animal viruses, which are unique in that they contain a linear single-stranded DNA genome (10). The classification of this family relies on morphology and functional characteristics.

Based on the ability to infect vertebrate or invertebrate cells the *Parvoviridae* family is divided into *Densovirinae* and *Parvovirinae* sub-families (10). The *Densovirinae* sub-family consists of three genera: *Densovirus*, *Iteravirus* and *Contravirus*. These parvoviruses are associated with insect hosts from the orders *Lepidoptera* and *Orthoptera*. The *Parvovirinae* sub-family (Table 1) includes the genera *Erythrovirus*, *Dependovirus* and *Parvovirus*. They are associated with mainly mammalian hosts. Only members of the *Dependovirus* and *Erythrovirus* genera are known to infect humans.

Parvovirus B19 is a member of the *Erythrovirus* genus and is the only member of the Parvoviridae family known to be pathogenic in humans. It is widespread, and manifestations of infection vary with the immunologic and hematologic status of the host. B19 is the cause of erythema infectiosum and acute symmetric polyarthropathy (45). It shows strict specificity for erythroid-lineage cells, due in part to the limited

distribution of its receptor, P antigen (11). So far, B19 is known to cause aplastic crisis in individuals with hemolytic disorder (71). B19 has also been linked with arthralgias, hydrops fetalis, and spontaneous abortions (79). Closely related viruses to B19 that cause similar diseases in primates have been proposed as additional members in the *Erythrovirus* genus (45).

Table 1. Classification of the *Parvovirinae* Sub-family

Genus	Virus	Natural Host
Erythrovirus	Parvovirus B-19	Human
	Parvovirus V9 [a]	Human
	Chipmunk parvovirus [a]	Chipmunk
	Rhesus parvovirus [a]	Rhesus monkeys
Dependovirus	Adeno-associated virus 1-6	Human
	Avian adeno-associated virus	Birds
	Canine adeno-associated virus	Dog
	Bovine adeno-associated virus	Cow
Parvovirus	Aleutian mink disease virus	Mink, skunk, raccoon
	Canine parvovirus	Dog
	Minute virus of mice	Mouse, rat
	Porcine parvovirus	Pig
	LuIII parvovirus	Unknown

[a] Proposed member of the genus

The Dependoviruses that comprises the adeno-associated viruses (AAV1-6) generally replicate in cells co-infected with a helper adenovirus or herpes virus (10). In the absence of their helper virus, the infecting AAV genome integrates into the host genome and remains latent. It integrates into a small region of human chromosome 19q13-qter (49). Rescue from the latent state requires co-infection with a helper virus or other stress, such as DNA damage (10).

Members of the *Parvovirus* genera, known as autonomous parvoviruses (APVs), are capable of infecting a variety of mammalian cell species. There is no evidence that the APVs integrate in the cell genome during lytic or persistent infections (10). They do not require a helper virus for replication but they do require cellular factors expressed during S phase or early G_2 phase of the host cell cycle (10).

Virion Properties

Until recently, parvoviruses were among the smallest DNA-containing viruses known capable of infecting mammalian cells (45). Virions are non-enveloped, containing a single copy of the viral genome encapsidated in an icosahedral protein capsid of about 18-26 nm in diameter with a molecular weight (MW) of 5.5-6.2 x 10^6 Daltons (10). Parvoviruses are among the most resistant viruses known. The capsid confers stability to the viral particles resisting inactivation by pH changes between 3 and 9, solvents such as alcohol and ether, and temperatures of up to 56 ºC for 60 minutes (10). Inactivation may be achieved by formalin, β-propiolactone and gamma irradiation. The infection by APVs, B19 and most parvoviruses is lytic and leads to the death with no evidence of integration (25, 43, 45, 56).

Genome Structure

The most characteristic feature of parvoviruses is their genome structure, consisting of an approximately 5 kb single-stranded, linear DNA molecule terminating in palindromic sequences which are folded back on themselves to form hairpin-like

structures that are stabilized by self-hydrogen bonding (Figure 1). The sequences of

the terminal palindromes, comprising <10% of the total genome, contain most of the

cis-acting signals necessary for viral DNA replication and encapsidation (3, 30, 72).

Figure 1. Palindromic Termini of Parvovirus LuIII. (A) DNA sequence forming the T-shaped secondary structure at the left (3' end) terminus of the minus strand of LuIII. (B) DNA sequence forming the U-shaped secondary structure (with the flip and flop conformations) at the right (5' end) terminus of the minus strand of LuIII (Taken from Diffoot *et al*., 1989).
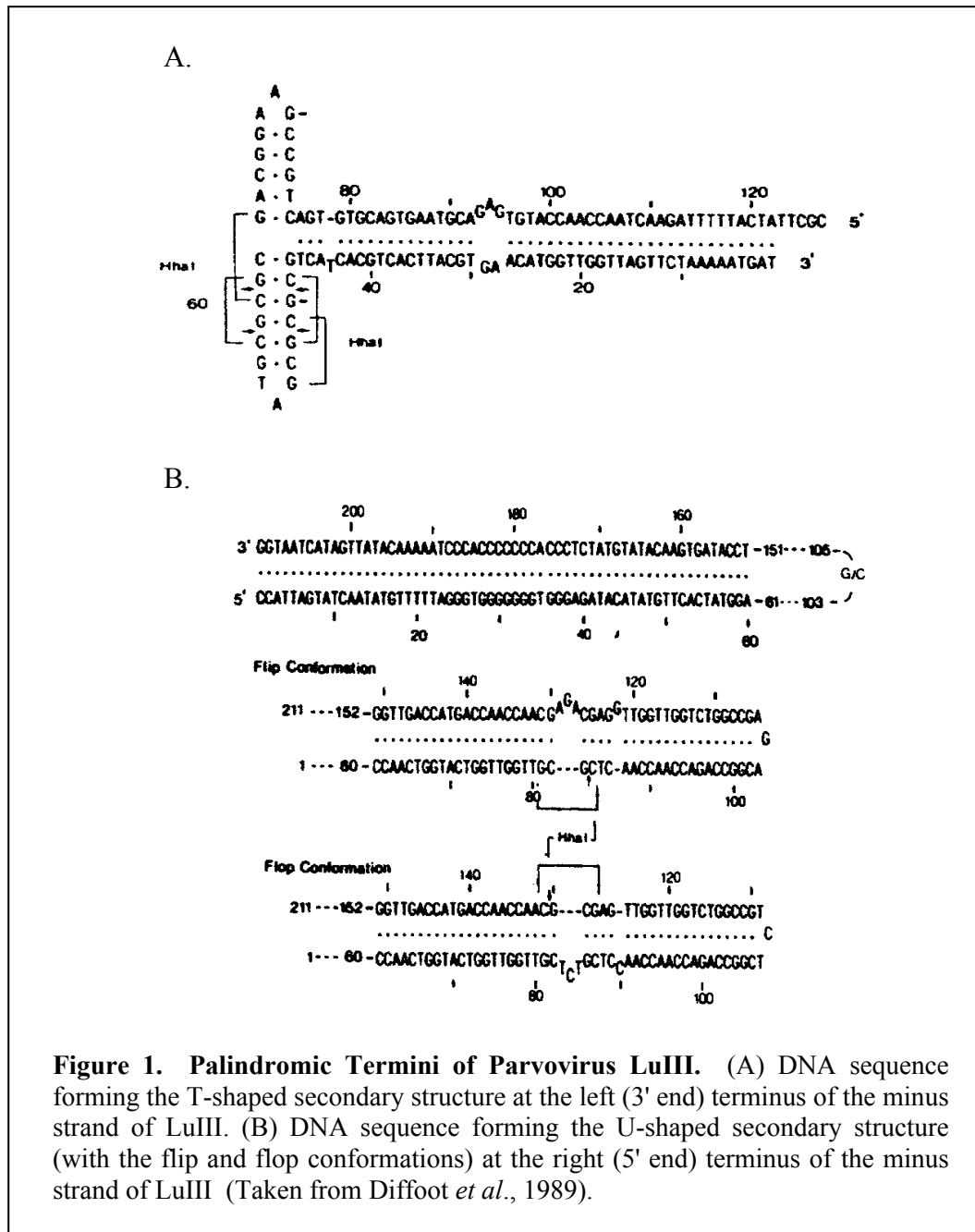
The AAV genome is flanked by identical yet inverted 145-nucleotide terminal repeat sequences forming a complex palindrome capable of folding into a T-shape hairpin structure (10). Like AAV, parvovirus B-19 has identical terminal repeats of 383 nt capable of assuming a T-shaped termini (45). In contrast to the genomes of AAV and B-19, the 3' and 5' hairpins of most of the APVs differ both in size and sequence. The right- and left-handed palindromes (5' and 3' end of the minus strand) are 200-211 and 115-122 nucleotides long, respectively (30). The right terminus can assume a T or U-shaped structure, while the left terminus has been observed only in the T- shaped conformation (30, 40).

The terminal palindromes also exhibit two alternative sequence orientations called *flip* and *flop* due to a few mismatched nucleotides (3). This mismatch results in a "bubble" within the stem of the palindrome, in which a triplet 5'-GAA in one arm of the palindrome is paired in the hairpin configuration to a doublet 5'-GA in the other arm (25). The right hairpin of the autonomous parvovirus MVM, HI and LuIII shows either *flip* (GAA paired to GA) or *flop* conformation (TTC paired to TC) (40). The left hairpin is found exclusively in the *flip* conformation (Figure 1) (30, 40).

Transcription

As for most animal parvoviruses, the coding strand of the APVs contains two major open reading frames (ORFs) under the control of two promoters, located at mu 4 (P4) and mu 38 (P38), respectively (13, 26) (Figure 2) (24, 41). The left hand ORF, under the control of the P4 promoter, codes for the two non-structural proteins NS1

and NS2 (27, 60) that are involved in viral DNA replication (26, 30) and cytotoxicity (13, 52). Frameshift mutations introduced in this ORF are lethal and prevent the excision and/or the subsequent replication of the viral DNA sequences (26). The right ORF of the APVs drives the synthesis of the various capsid polypeptides VP1, VP2 and VP3, all under the control of P38 promoter (10).

B19 has only one promoter at mu 6 (P6) that drives both the synthesis of the major nonstructural protein NS1 and the capsid proteins VP1 and VP2 (10, 45). AAV has three promoters located at mu 5 (P5), 19 (P19) and 40 (P40) (10). Promoter P5 and P19 drives the synthesis of four nonstructural proteins, known as the Rep proteins. The capsid proteins are under the control of P40 promoter.

Figure 2. Transcription Map of most Autonomous Rodent Parvoviruses. The genome is organized into two open reading frames (ORF). The left ORF encodes for the nonstructural proteins (NS) under promoter P4 and the right ORF encodes for the structural proteins (VP) under promoter P38 (Adapted from Naeger *et al.*, 1990).
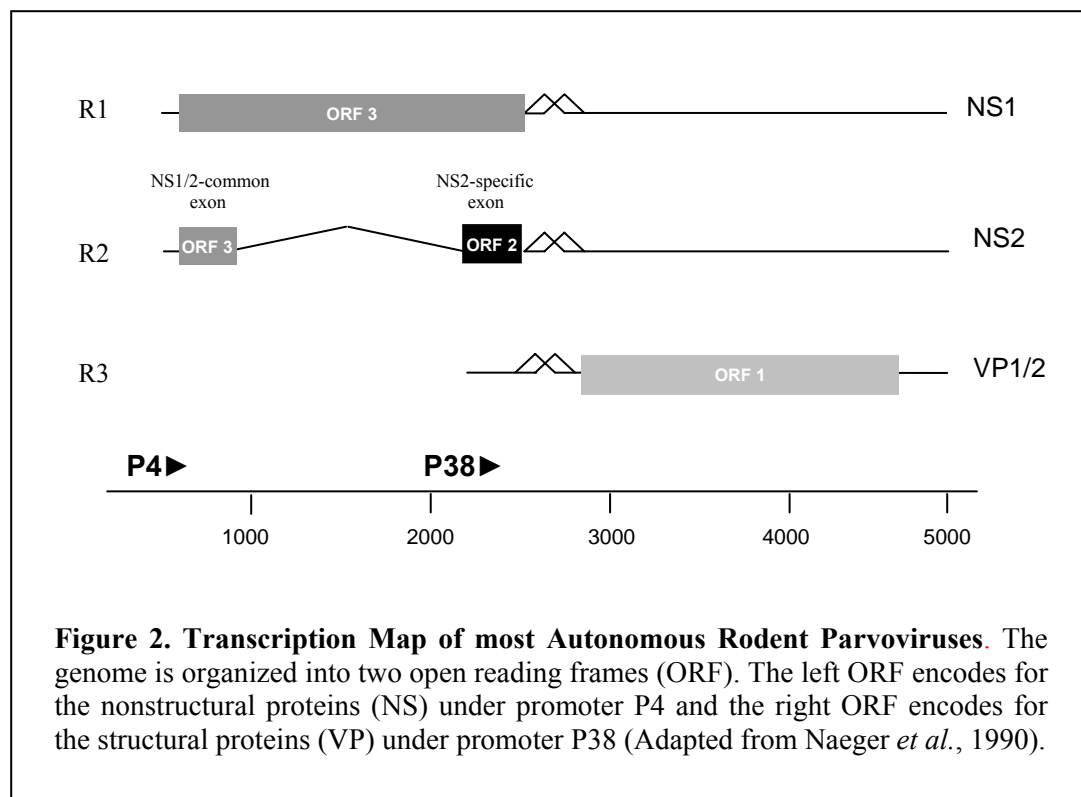
Viral Proteins

NS1 is the major non-structural protein of the APVs and is essential for a productive infection in all cell types (14, 52, 54). It is an abundant and long-lived nuclear phosphoprotein of 83 kDa with 3' -to- 5' helicase (21) and ATPase activities (78). NS1 serves as an initiator of viral DNA replication (26, 29, 31, 34) and its function is dependent on protein kinase C phosphorylation (38, 65, 66, 67). It is a site-specific DNA binding protein, recognizing the sequence $(ACCA)_2$ present in the viral origins, and initiates replication by introducing a single-stranded nick at a specific sequence located close to the core recognition site (35, 77). NS1 also functions as a transcriptional activator of both MVM P4 and P38 promoters (17, 35, 53). In addition to its function in the viral cycle, NS1 was also shown to inhibit the growth of transformed cells (13, 52) by a yet unknown mechanism.

NS2 is a 25-kDa protein mainly located in the cytoplasm and shares a common amino-terminal domain with NS1, which comprises the first 85 amino acids of each protein (30). It is only required for productive replication in cells of murine origin (63). In these cells NS2 influences multiple steps in the viral life cycle by currently undefined mechanisms (14, 63, 64). One of these steps is involved in capsid assembly; in its absence DNA replication is severely diminished resulting in undetectable single-strand progeny (36).

The non-structural proteins of AAV are involved in replication and site-specific integration of the AAV genome. Rep 68 and Rep78, have been shown to have

site-specific DNA binding, nicking, and transcriptional activities, as well as ATPase and helicase functions (10). Rep 40 and Rep52 are important for the production of single-stranded DNA progeny. The function of the B19 nonstructural protein NS1 is not fully understood, but based on data from other parvoviruses, similar functions have been suggested (45).

The capsid proteins of most autonomous parvoviruses, VP1 and VP2, have a molecular weight of nearly 83 and 64 kDa, respectively (10, 25, 74). The major capsid protein is VP2. The amino acid sequences of VP1 and VP2 are identical except for a region at the amino terminus that is unique for VP1 (43). A third polypeptide VP3, with approximately 62 kDa, can be produced from the proteolytic cleavage of VP2 (10). The B19 capsid is composed of VP1 and VP2, with a molecular weight of 84 and 58 kDa, respectively. Dependovirus AAV has the three coat proteins VP1, VP2 and VP3. They range in size from 62 to 87 kDa (10).

Parvovirus DNA Replication

Parvoviral DNA replication is a complex process, involving the formation of hairpin structures, extension to form a complete double-stranded replicative form (RF), endonuclease cleavage, and repetition of these events. It proceeds by a rolling hairpin mechanism where the replication fork is flipped back and forth along the linear genome by sequential synthesis and rearrangement of the palindromic viral termini generating long, palindromic, duplex concatamers (Figure 3) (3, 31, 55). Autonomous parvovirus replication and assembly occurs in the nucleus and is dependent upon host

enzymes and cellular functions occurring during the S phase of the cell cycle (10, 29, 44). Active sites of viral genome replication and viral capsid assembly following infection have been identified and termed autonomous parvovirus-associated replication (APAR) bodies (8, 81).

Figure 3. **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 monomeric RF; reRF, rabbit-eared RF; v, viral strand; c, complementary strand. The open polygon in step 6 represents the capsid (Taken from Kuntz-Simon *et al.*, 1999).
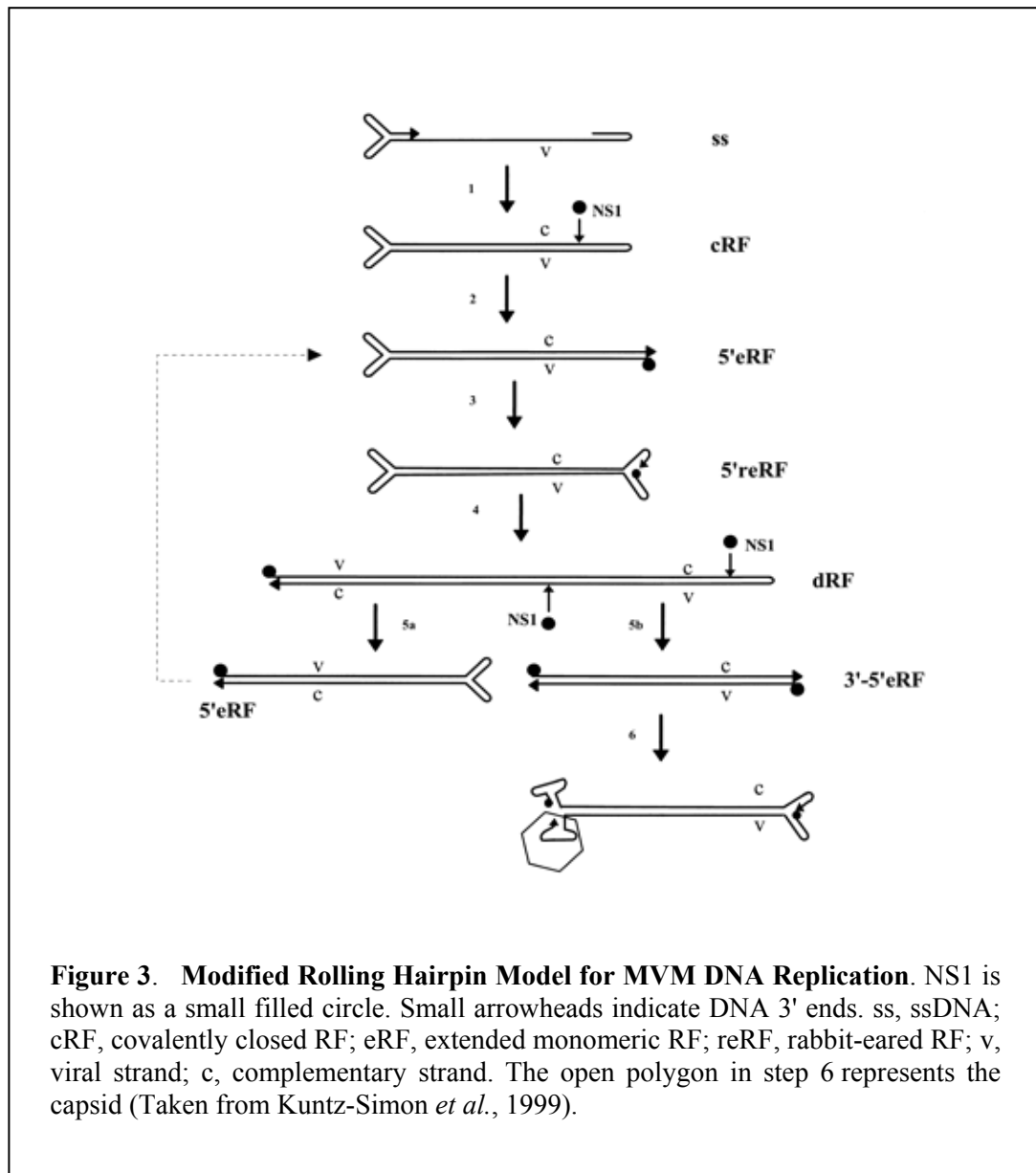
MVM has been studied as a model for the autonomous parvovirus DNA replication (43). Replication initially proceeds rightward from the terminal 3' hydroxyl of the hairpin stem (Figure 3). The 3' hairpin serves as a primer, which allows a host polymerase to synthesize a complementary copy of the internal sequence of the viral genome until the growing strand reaches the folded back 5' terminus at the right end, resulting in a covalently closed DNA replicative form (cRF) (step1) (6). Further processing involves the opening of the cRF at its right end by NS1 becoming covalently attached to the 5' at the nick site via a phosphotyrosine bond (26), followed by displacement and copying of the right end hairpin, giving rise to an extended molecule designated 5' eRF (step 2) (3, 26, 77). Rearrangement of the copied right hand palindrome into hairpin structures creates the so-called rabbit-eared replicative form (5' reRF) (step3) (50). This provides a primer for strand-displacement synthesis, leading to the formation of a dimer duplex intermediate (dRF) in which two unit-length copies of the genome are joined by a single duplex copy of the original 3' palindrome (step 4) (6, 30, 77, 78).

In the bridge arrangement of the dRF the mismatched doublet GA and triplet GAA are now based paired to their complementary sequences (Figure 4). The sequence surrounding the doublet is a potent origin, but the analogous region containing the triplet is completely inactive (29). The actual sequence of the GA doublet is unimportant, but insertion of any third nucleotide here inactivates the origin, indicating that it represents a critical spacer element (29).
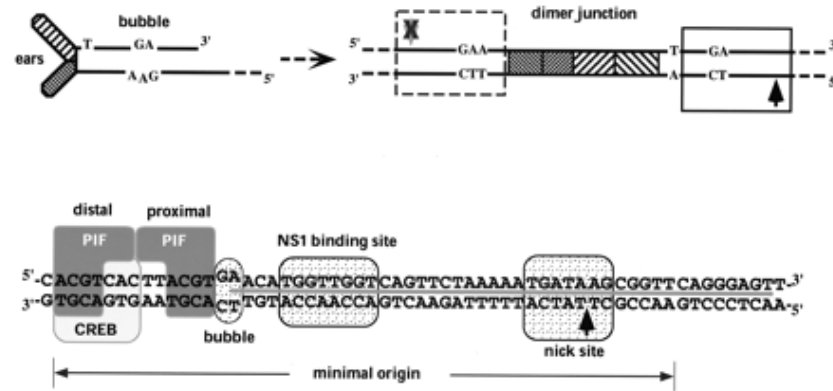
Figure 4. Formation and Organization of the MVM Origin of Replication. (Top left) Structure of the left-end hairpin showing the 3' OH used for priming replication and the mismatched bubble sequence. (Top right) Organization of the left-end hairpin sequences within the duplex dimer junction generated by replication through the hairpin. Hatched boxes represent the palindromic sequences. The boxed sequence (expanded below) represents the minimum active replication origin in the outboard arm, while the sequence in the dashed box represents the inactive origin on the inboard arm. The potential nick sites on each side of the junction are indicated by arrows (black arrows, active site: grey arrow with X, inactive site). (Bottom panel) Sequence of the active origin showing the different elements involved in replication. The solid line between the two strands of DNA sequence indicates the region protected by NS1 from DNAse digestion (Taken from Christensen *et al.*, 2001).

The junction region thus formed contains an active NS1 driven origin (34, 56). Figure 4 illustrates how the sequence of the 3' terminus becomes rearranged in the dimer junction. Genetic mapping studies revealed that the minimal active MVM-3' replication origin is a multi-domain structure of approximately 50 bp sequence derived from the outboard arm of the palindromic dimer bridge structure (29, 30, 34). It contains three distinct recognition elements: an NS1 binding site $(ACCA)_{1-3}$; an NS1

nick site (CTWW↓TCA- where W designates A or T); and a region containing a consensus activated transcription factor (ATF/CREB) binding site, essential for origin activity. NS1 binds the minimal origin in an ATP-dependent manner but is unable to initiate replication by nicking the DNA (18).

A cellular factor termed PIF, for parvovirus initiation factor, acts as an essential cofactor for NS1 in the replication initiation process allowing efficient and specific nicking of the 3' minimal origin and leaving NS1 covalently attached to the 5' end of the DNA at the nick site (18, 19). PIF recognizes an ACGT motif within the ATF consensus in conjunction with a second ACGT motif located nearer the NS1 binding site, but it is not a known member of the ATF family (19). It is a widely expressed heterodimer consisting of two related polypeptides, p79 and p96, but only p79 activates the NS1 nickase function (20). The region containing the PIF binding site is highly conserved in the 3' hairpin of other parvoviruses related to MVM, such as LuIII, H1 and MPV, and to a lesser degree in several others including FPV and CPV (18).

Once the dimer junction is formed, it is resolved asymmetrically by NS1 which introduces a single-stranded nick into the active origins generating two types of replicative form DNA: an extended palindromic form, and a turnaround form that recreates the left-hand termini (Figure 3, steps 5a and 5b) (33, 34, 55). The turnaround molecule generated in this way re-enters the cycle, while the extended molecule is

thought to lead to the displacement of single-stranded genomic DNA, which is then packaged into pre-formed empty capsids (step 6) (62).

In vitro reconstitution of replication has identified that proliferating cellular nuclear antigen (PCNA), a ring-shape homotrimeric protein forming a sliding clamp at the primer-template junction, and single-stranded DNA binding protein (RPA) are absolutely required (18). Recently, a molecular model of the parvovirus replication fork was proposed (Figure 5). It includes various components of the leading-strand synthesis machinery: PCNA, RPA, Pol δ (48) and the replication factor C (RFC), the PCNA loading factor (21). The model proposes that these factors, together with NS1, form the parvoviral replication fork.

Figure 5. 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) (Taken from Christensen *et al.*, 2001).
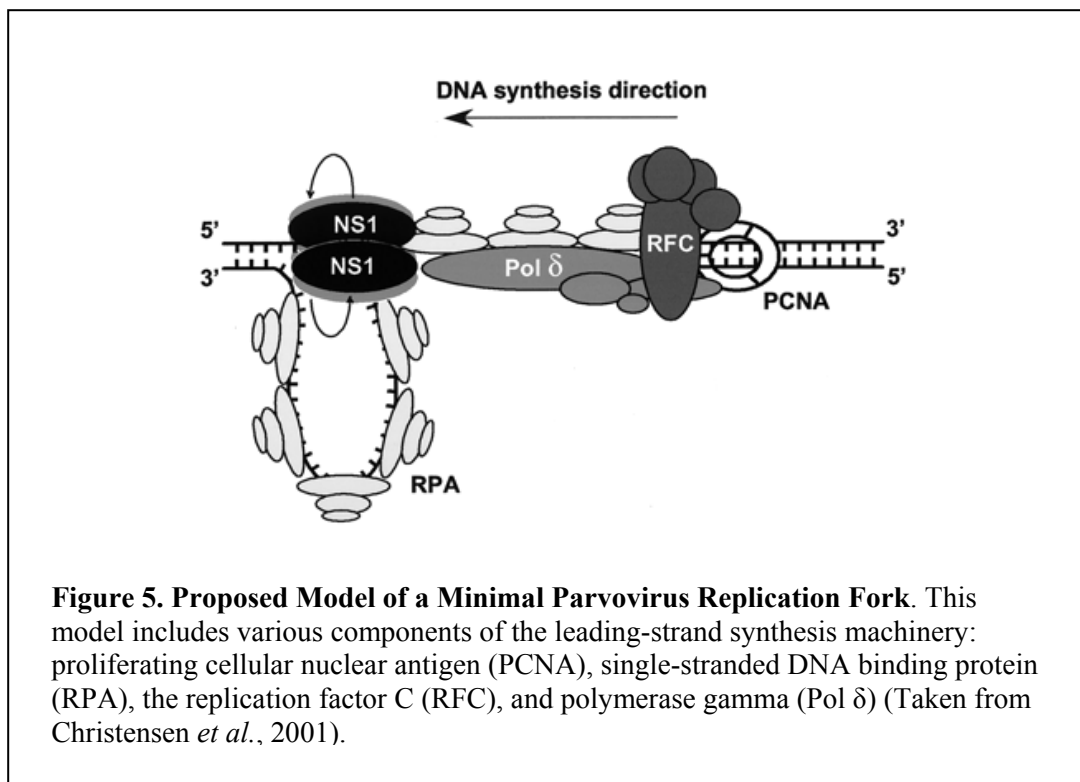
Although the two viral telomeres are very different from each other in size, primary sequence and secondary structure, they both contain elements that finally become rearranged to create an NS1 dependant origin of replication, activated by different cellular cofactors. Sequences from the left-end telomere form a functional origin only on one side of the dRF intermediate (29, 34). In contrast, the right-end origin can operate in its cRF hairpin configuration and as a fully duplex linear sequence derived from either arm of a palindromic tetramer intermediate (32, 37). Unlike PIF heterocomplex, the essential cofactor for the right end origin is a non-sequence-specific DNA-binding protein from the high-mobility group 1/2 (HMG 1/2) family of chromatin-associated polypeptides (32).

Encapsidation

The synthesis and packaging of single-stranded progeny viral DNA are simultaneous processes which require the presence of preformed empty viral capsids (62). It was shown that the 3' end of the MVM DNA in its hairpin configuration binds to empty capsids (75). The binding is due to the capsid protein VP1, who was found to discriminate between the extended and hairpin forms of the 3' terminus, binding only to the latter. However, it is still not clear how this interaction can lead to DNA encapsidation.

Studies suggest that the *cis* signals required for the encapsidation reside in the genomic termini, since molecules containing only the termini sequences were packaged (72). Parvoviruses can encapsidate either plus or minus strand, however the

ratio varies among members (Table 2). The mechanisms underlying strand-packaging process are not yet understood. Based on AAV, it was thought for a time that parvoviruses containing identical palindromic sequences at either end of the genome packaged either strand, while parvoviruses containing distinct termini packaged predominantly the minus strand (2, 4, 71).

Nevertheless, evidence indicates that identical termini are not required for equal encapsidation of plus and minus strand. Bovine parvovirus (BPV) with unique 3' and 5' termini, encapsidates 10% of the plus strand (15). Moreover, the autonomous parvovirus LuIII, like AAV and B-19, encapsidates equal amounts of plus and minus (9, 10, 40, 62), yet its terminal sequences share over 90% homology with the termini of the rodent parvoviruses which encapsidate 99% minus strand (41).

Table 2. Encapsidation Pattern of Selected Parvovirus

Sub-family	Parvovirus	Terminal hairpin	Encapsidation Pattern
Dependovirus	AAV	Identical	50 % (+), 50 % (-)
Erythrovirus	B19	Identical	50 % (+), 50 % (-)
Parvovirus	H1	Non-identical	99 % (-)
	MMV	Non- identical	99 % (-)
	LuIII	Non-identical	50 % (+), 50 % (-)

Comparison of the sequences of LuIII, MVM and H1 reveals the presence of adenine-thymine (A/T) rich sequence near the right end that may be determinant of the encapsidation pattern for LuIII (41). This region is 47 bases long, beginning at nucleotide 4560 of the LuIII genome, and is not present in H1 virus or MVM. It has been postulated that this sequence might be responsible for the ability of LuIII to package both strands of its genome with equal frequency. It is also possible that the A/T rich region can also function as an autonomously replication sequence (ARS) as those found in yeast (unpublished data).

Parvoviruses for Gene Therapy

Viruses are naturally evolved vehicles that efficiently transfer their genes into host cells. This ability has led to the widespread use of viruses as genetic vectors. Parvoviruses display several characteristics that make them suitable as viral vectors (43, 59). Recombinant vectors based on parvoviruses are derived by substituting foreign genes for the entire or some coding sequences. This could be achieved by the co-transfection of one plasmid containing the transgene between the terminal sequences, and a defective helper construct containing the viral structural and nonstructural genes (57, 59). Retention of the terminal palindromes is necessary, and is generally considered sufficient for efficient parvoviral replication and encapsidation of progeny single-stranded DNA when the helper functions are provided in *trans* (3, 30, 72).

AAV based vectors have received a significant interest for cancer gene therapy. Some of the major advantages of AAV vectors include a stable integration in a site-specific way, long-term expression of the transgene suitable for human applications, and low immunogenicity (7, 67, 68). In 1996, two separate publications demonstrated long-term expression of the marker protein β-Gal (79) and the secreted protein erythropoietin (46) in muscle tissue after a single injection of recombinant molecules of AAV virus. These studies initiated the efforts by numerous laboratories in evaluating AAVs potential *in vivo*, resulting in near identical results in muscle, lung, gut, liver, CNS and eye (61).

The autonomous parvoviruses also have received considerable interest as potential vectors for gene therapy. Rodent parvovirus H-1, MVM and LuIII are the only autonomous parvovirus that have been exploited for use as vehicles for gene transfer given that they show a broad cell tropism particularly for those cells expressing a neoplastically transformed phenotype, as well as a high efficiency of transduction with no disease association (1, 59, 47). Such vectors show promise for the delivery of therapeutic genes in situations requiring cell-specific, short-term expression.

MVM vectors have been successfully used for the transduction of the chloramphenicol acetyltransferase (CAT) reporter gene into a variety of normal and transformed human cells, including fibroblast, epithelial cells, T lymphocytes, and macrophages (42). The reporter gene was inserted into the capsid-encoding region,

under the control of P38 promoter. Russell *et al* have also demonstrated transduction of human interleukin 2 (IL-2) and murine interleukin 4 (IL-4) from cDNA substituted for capsid sequences in an infectious clone of MVM viral genome on non-transformed fibroblasts of rodent and human origin (69).

Maxwell and colleagues have developed several vectors systems based on LuIII genome, wherein the luciferase and ß-galactosidase reporter genes replace the viral coding sequences. These vectors transduced either the *lacZ* gene or luciferase under the control of the viral constitutive P4 promoter into a variety of human cells co-transfected with a defective virus construct that supply viral helper functions in *trans* (23, 57). Further recombinant molecules were constructed replacing the P4 promoter with a liver-specific promoter and also with inducible promoters such as GAL4 and the bacterial tetracycline repressor showing that LuIII vectors can be potentially useful for gene transfer applications requiring the restriction of expression to specific tissues or cell types (58).

Recently, a vector based on a chimera between MVM and LuIII that expresses the *Borrelia burgdorferi* outer surface protein A (OspA) instead of the coat proteins, was constructed and successfully used in mice as a non-proliferating vaccine (67). Vaccination with a single vector dose elicited a high-titer anti-OspA-specific antibody, inducing both humoral and cell-mediated immune response.

CHAPTER III

Materials and Methods

The 3' LuIII 3' minigenome (Figure 6) has two copies of the left end palindrome of the autonomous parvovirus LuIII (nt 1-277), cloned into the *Eco*RI site of pUC19 (38). However, this clone has an insertion of unknown sequence of approximately 300 nt between the two termini. To construct a clone with only two copies of the left end, three different strategies were followed. The first two strategies consisted in eliminating the stuffer sequence by digesting the plasmid independently with two different restriction enzymes, and then re-circulating it with T4 DNA ligase. The third strategy consisted in constructing the recombinant molecule *de novo*.

Figure 6. Map of 3' LuIII 3'. Restriction sites and the nucleotide position in its respective sequences are indicated (De Jesús, 2000).
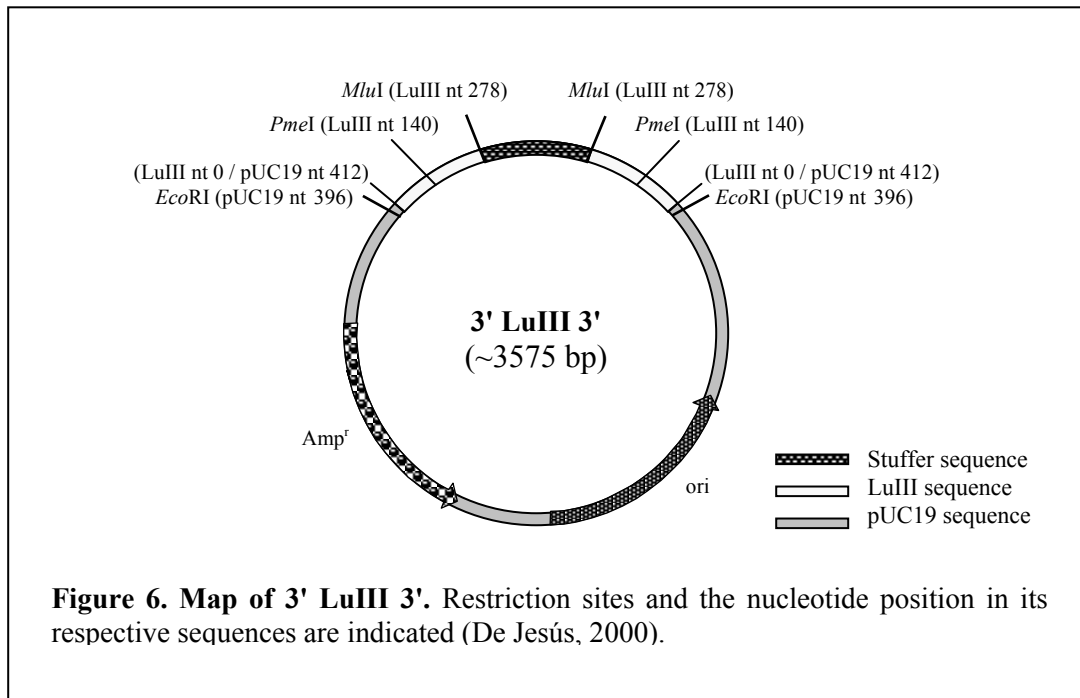
Elimination of the stuffer sequence between the left termini of 3' LuIII 3'

There are four different restriction sites within the hairpin that can be used to eliminate the stuffer sequence of 3' LuIII 3': two *Pme*I sites located at nt 140 of the LuIII sequence, and two *Mlu*I sites located at nt 278 of the LuIII sequence (Figure 7). The construction was digested independently with *Pme*I (GTTT/AAAC) (New England Biolabs, Beverly, MA) and *Mlu*I (A/CGCGT) (Roche Applied Science, Indianapolis, IN) at 37 ºC for one hour and then electrophoresed on a 1.2% agarose gel in 1X TBE (100mM Tris-borate, 2mM EDTA, pH 8.3) buffer at 76 V.

The restriction enzyme *Pme*I cuts 3' LuIII 3' at nt 140 of the LuIII sequence generating two fragments of ~576 and 2998 bp (Figure 8). *Mlu*I cuts the clone at nt 278 of the LuIII sequence generating two fragments of ~300 and 3274 bp, respectively (Figure 9). The fragments of 2998 (from the *Pme*I digestion) and 3274 bp (from the *Mlu*I digestion) corresponding to the pUC19 sequence containing both copies of the left terminus of LuIII were purified using the Geneclean Spin Kit® (QBio-gene, Carlsbad, CA) and then subsequently run on a 1% agarose gel in 1X TBE at 76 V to confirm the nature of the desired fragments (Figure 10).

Approximately, 200 ng of each fragment were re-circularized using T4 DNA ligase (Roche Applied Science, Indianapolis, IN). The fragment generated by *Pme*I (2998 bp) was ligated overnight at room temperature with 5U of T4 DNA ligase, and the fragment generated by *Mlu*I (3274 nt) was ligated overnight at 4 ºC with 1U of T4 DNA ligase, and subsequently transformed into competent cells by heat-shock (5).
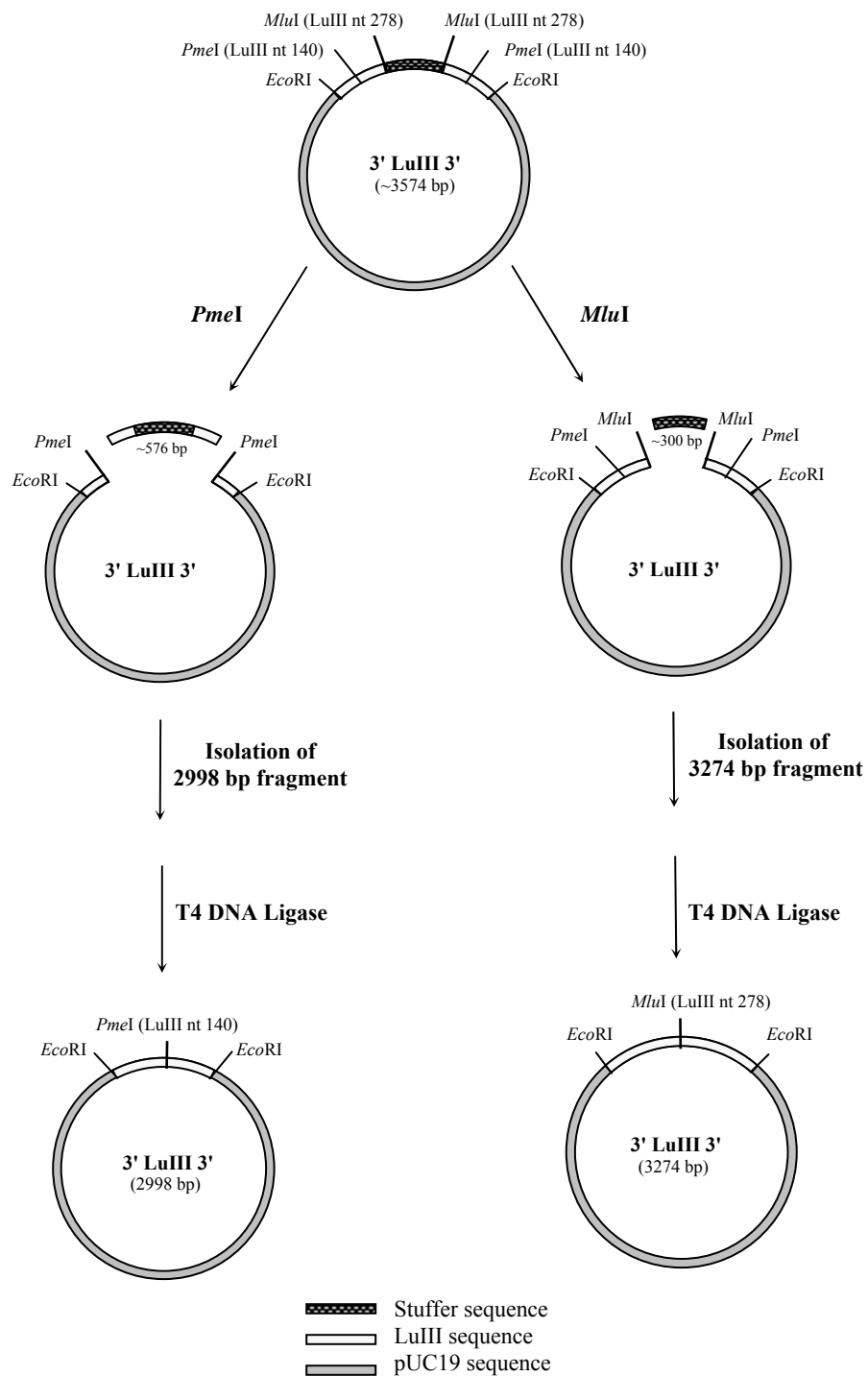
Figure 7. Strategy used to eliminate the stuffer sequence between the two left end termini of 3' LuIII 3'. Restriction sites and the nucleotide position in its respective sequences are indicated.
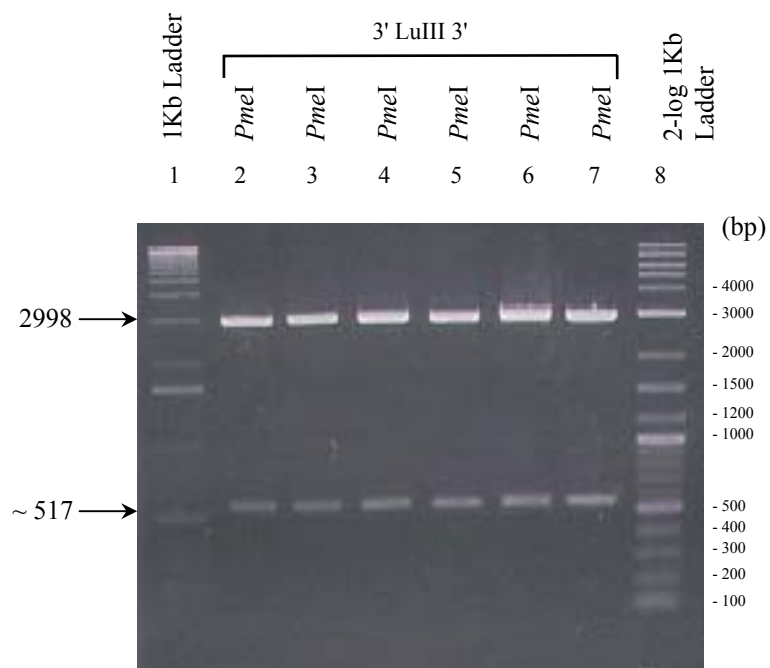
Figure 8. Digestion of 3' LuIII 3' with *Pme*I. Lanes 2-7: *Pme*I digestions of 3' LuIII 3'. The digestions were electrophoresed on a 1.2% agarose gel in 1X TBE at 76 V. Sizes of the 1Kb DNA Ladder (lane 1) (Gibco, Gaithersburg, MD) and 2-*log* 1Kb DNA Ladder (lane 8) (New England Biolabs, Beverly, MA) are indicated.
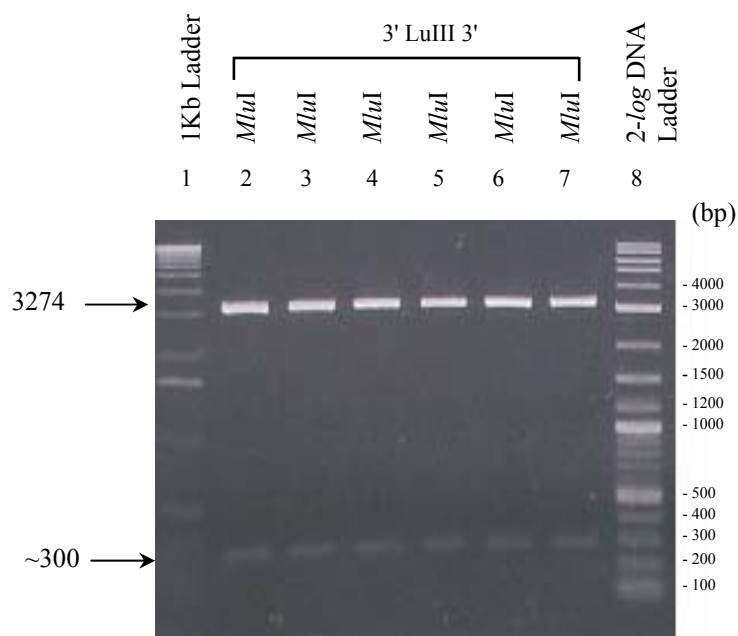
Figure 9. Digestion of 3' LuIII 3' with *Mlu*I. Lanes 2-7: *Mlu*I digestions of 3' LuIII 3'. The digestions were run on a 1.2% agarose gel in 1X TBE at 76 V. Sizes of the 1Kb DNA Ladder (lane 1) (Gibco, Gaithersburg, MD) and 2-*log* 1Kb DNA Ladder (lane 8) (New England Biolabs, Beverly, MA) are indicated.
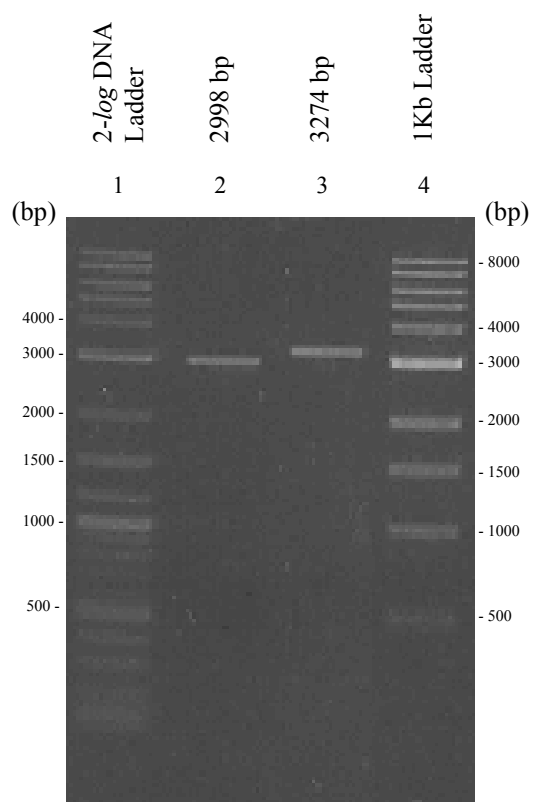
Figure 10.   Isolation of the 2998 and 3274 bp fragments. Lanes 2-3: purified fragments of 2998 and 3274 bp generated by the *Pme*I and *Mlu*I digestion, respectively. Samples were run on a 1% agarose gel in 1X TBE at 76 V. Sizes of the 2-*log* 1Kb DNA Ladder (lane 1) (New England Biolabs, Beverly, MA) and 1Kb DNA Ladder (lane 4) (New England Biolabs, Beverly, MA) are indicated.

Construction of the recombinant molecule 3' LuIII 3' *de novo*

Attempts to clone two copies of the 3' hairpin sequences into the *Bam*HI site of pUC19 were also made. The 3' hairpin of LuIII was obtained from pGLu883 (40), a full-length genomic clone of LuIII (5135 nt) cloned into the pUC19 vector (Figure 11). pGLu883 was digested with both *Bam*HI (G/GATCC) (Roche Applied Science, Indianapolis, IN) (pUC19 nt 417) and *Mlu*I (LuIII nt 278) for two hours at 37 ºC and then electrophoresed on a 1.2% agarose gel in 1X TBE buffer at 75 V (Figure 12).

Figure 11. Map of pGLu883. pGLu883 is a genomic clone of parvovirus LuIII. Restriction sites and the nucleotide position in its respective sequences are indicated (Diffoot *et al*, 1993).

Figure 12. Strategy used to clone two copies of the left end palindrome of LuIII into pUC19 *de novo*. Restriction sites and the nucleotide position in its respective sequences are indicated.

The *Bam*HI/*Mlu*I digestion generated three fragments of approximately 284, 2686, and 4861 bp (Figure 13). The 284 bp fragment contains the left end sequence of LuIII (nts 1-278), the 2686 bp fragment corresponds to the pUC19 vector sequence, and the 4861 bp fragments contains nts 279-5135 of the LuIII sequence. The 284 bp fragment corresponding to the left end hairpin was isolated and purified as previously described and confirmed on a 1.2% agarose gel run in 1X TBE buffer at 60 V (Figure 14).

The 284 bp fragments were ligated through the *Mlu*I site in an overnight reaction at 4 ºC using 1U of T4 DNA ligase. The ligation was digested with *Bam*HI generating a fragment of 568 bp corresponding to the two copies of the 3' hairpin in a "head to tail-tail to head" conformation (nts 1-278, 278-1). The digestion reaction was then electrophoresed on a 1.2% agarose gel in 1X TBE buffer and the fragment generated was purified as previously described (Figure 15).

The purified product was ligated into the *Bam*HI site of pUC19, that was previously treated with calf intestinal alkaline phosphatase (CIAP) (Roche Applied Science, Indianapolis, IN) for one hour at 37 ºC. Approximately, 50 ng of pUC19 and 150 ng of the insertion were ligated in an overnight reaction at 4 ºC with 1U of T4 DNA ligase. and the possible recombinant molecules were transformed into competent cells.
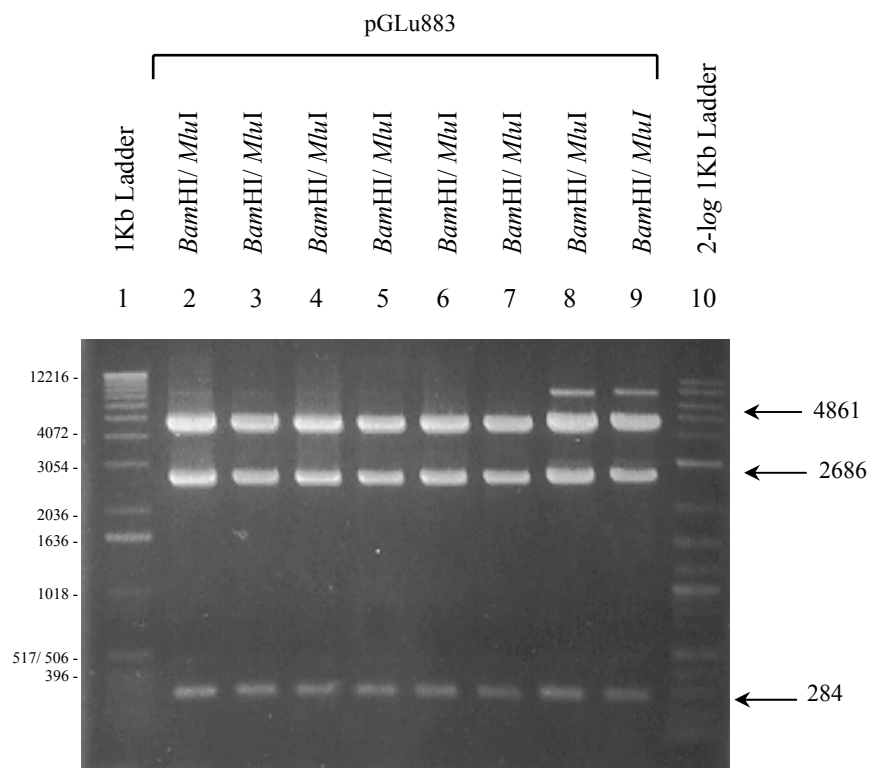
Figure 13. Digestion of pGLu883 with *Bam*HI and *Mlu*I. Lanes 2-9: *Bam*HI/*Mlu*I digestions of pGLu883. The digestions were run on a 1.2% agarose gel in 1X TBE at 75 V. Sizes of the 1Kb DNA Ladder (lane 1) (Gibco, Gaithersburg, MD) and 2-*log* 1Kb DNA Ladder (lane 10) (New England Biolabs, Beverly, MA) are indicated.
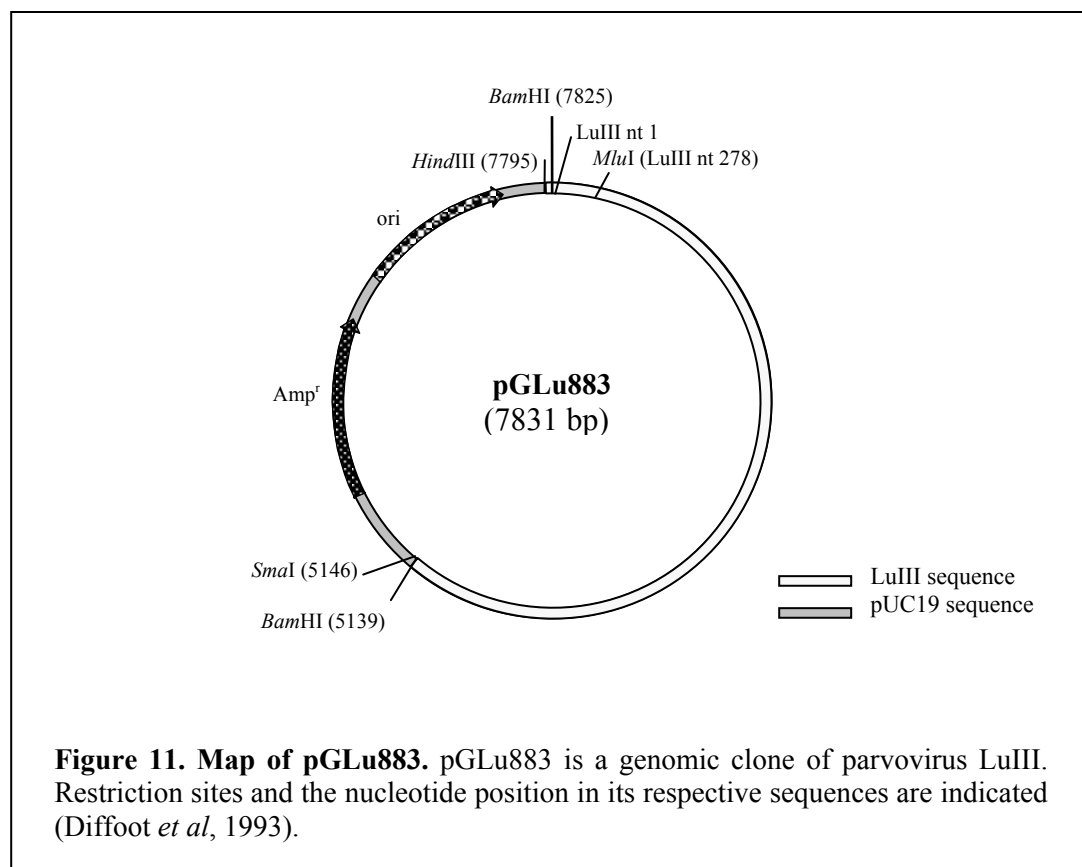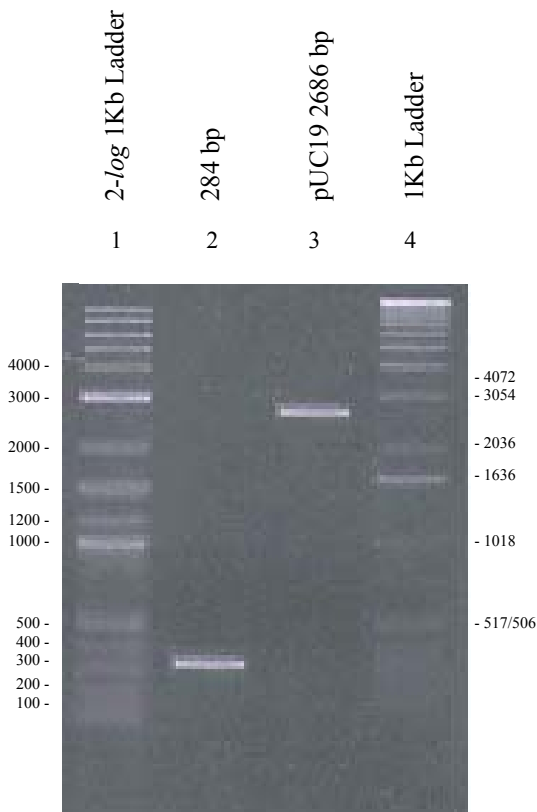
Figure 14. Purified fragment containing the left end sequence of the LuIII virus. Lane 2: isolated LuIII fragment of 284 bp. Lane 3: vector pUC19 (*Bam*HI-CIAP). The samples were run on a 1.2% agarose gel in TBE buffer at 60 V. Sizes of the 2-*log* 1Kb DNA Ladder (lane 1) (New England Biolabs, Beverly, MA) and 1Kb DNA Ladder (lane 4) (Gibco, Gaithersburg, MD) are indicated.
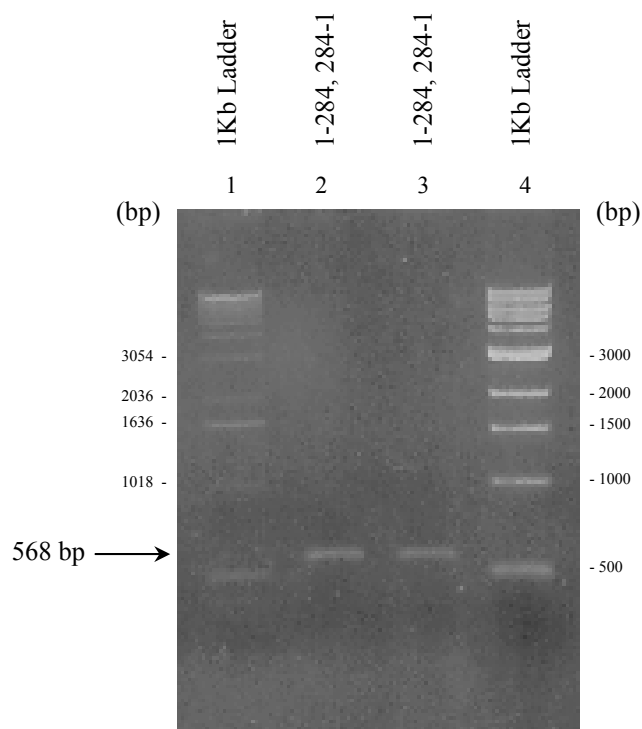
Figure 15. Purified fragments containing two copies of the left end hairpin of LuIII. Lanes 2-3: 284 bp fragments ligated *in vitro* and then digested with *Bam*HI generating a fragment of 568 bp. The digestion was electrophoresed on a 1.2% agarose gel in TBE buffer at 70 V. Sizes of the 1Kb DNA Ladder (lane 1) (Gibco, Gaithersburg, MD) and 1Kb DNA Ladder (lane 4) (New England Biolabs, Beverly, MA) are indicated.
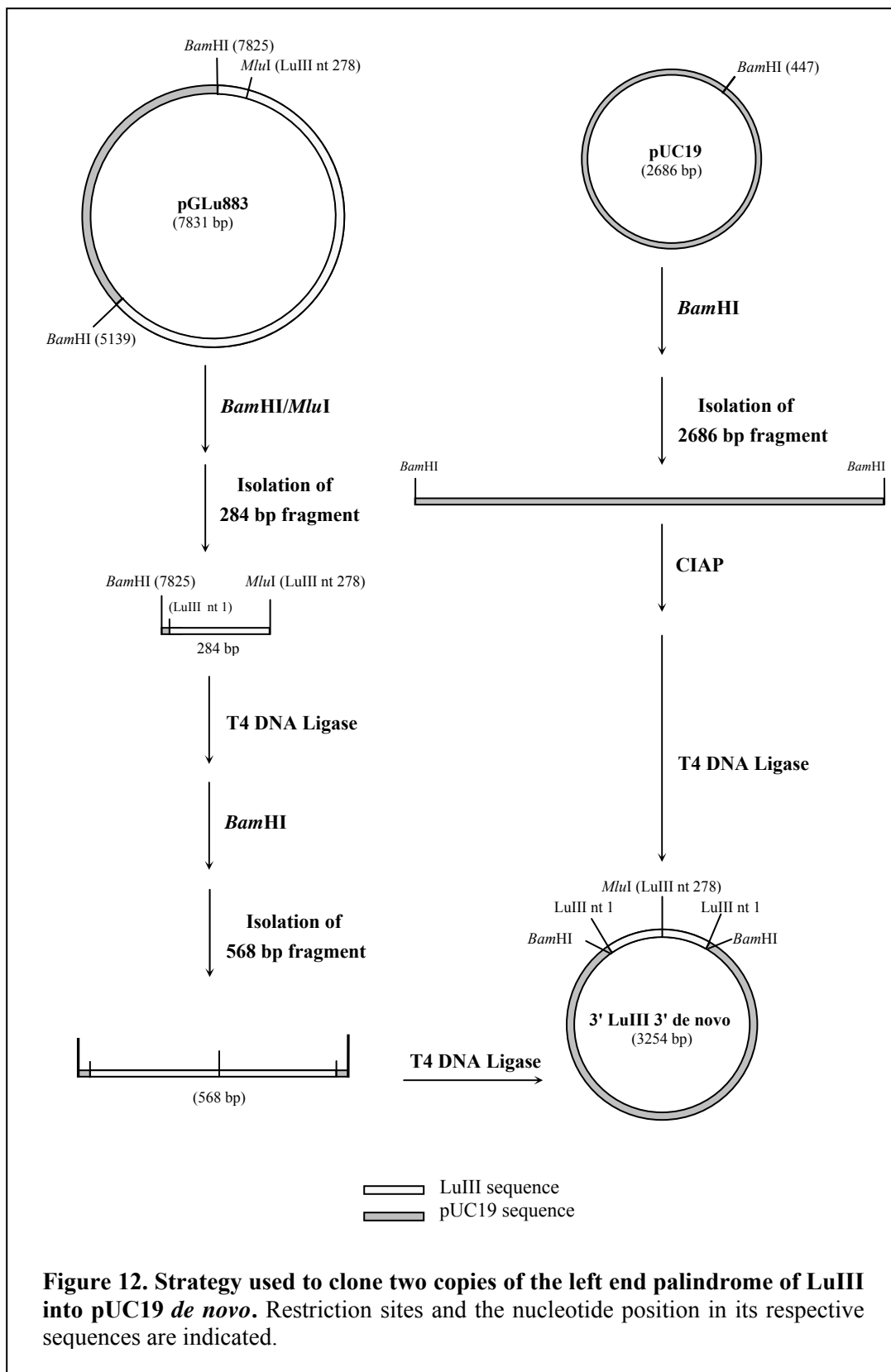
Preparation of Competent Cells

Two different strains of *Escherichia coli* were used as competent cells: DH5α

[(*lac*ZΔM15 Δ *(lac*ZYA-argF*) rec*A1 *end*A1 *hsd*R17 ($r_k m_k^+$) *pho*A *sup*E44 *thi gyr*A96

*rel*A1)] (ATCC, Rockville, MD) and SURE®2 (Stop Unwanted Rearrangement

Events) supercompetent cells [(e14⁻ *(McrA⁻) Δ (mcrCB-hsd SMR-mrr) 171 endA1*

*supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::*Tn5 (Kan^r) *uvrC* [F' *proAB*

lacI^qZΔM15 Tn*10* (Tet^r) Amy Cam^r]] (Stratagene, La Jolla, CA). The latter strain was

designed to facilitate cloning of DNA containing irregular structures such as inverted

repeats or secondary structures, which is the case of the left end hairpin of LuIII.

Competent cells were prepared by the calcium chloride method (5). Briefly, 1

mL from a freshly overnight culture was transferred to 100 mL LB (10 g tryptone, 5 g

yeast extract, 5 g NaCl, 1 ml 1N NaOH *per liter*) broth and grown for approximately

three hours or until an OD_{600} of 0.3-0.4 was reached. Cells were harvested by

centrifugation at 2500 rpm for 10 minutes at 4 ºC. The pellet was resuspended gently

in 25 ml of cold 50 mM $CaCl_2$ and placed on ice for 45 minutes. Cells were

centrifuged as before and then resuspended gently in 5 mL of cold 50 mM $CaCl_2$-20%

glycerol. Aliquots of 100 µL of competent cells were made and stored at - 80 °C.

Transformation of Competent Cells

The recombinant molecules were transformed in both DH5α and SURE®2

competent cells. The control samples are shown in Table 3. Competent cells were

thawed on ice for 15 minutes. The DNA was added to the cells and incubated on ice

for 30 minutes. Cells were heat-shocked in a 42 ºC water bath and subsequently
incubated on ice for 2 minutes. DH5α cells were heat-shocked for 2 minutes and
SURE®2 competent cells were heat-shocked for 30 seconds. Then, 100 μL of pre-
heated (42 ºC) LB broth was added to both cell samples and then incubated at 37 ºC
for 1 h with shaking at 225 rpm. DH5α transformed cells were spread on LB agar
plates containing 50 mg/mL ampicillin and 80 μL of 2% X-gal. SURE®2 transformed
cells were spread on LB plates containing 50 mg/mL ampicillin, 100 μL of 2% X-gal
and 100 μL of 10 mM IPTG. The sample used for control of cell growth was grown on
plates without ampicillin. All the plates were incubated overnight at 37 °C.

Table 3. Transformation of ligations for the recovery of the possible 3' LuIII 3' plasmids

A. Elimination of Stuffer Sequence Strategy

Sample	DNA Added	Plate	Concentration of DNA (ng)
1	None	Control for Cell Growth	0
2	None	Control for Antibiotic	0
3	pUC19	Efficiency of Transformation Control	~20
4	Re-circulated 2998 bp fragment	Experimental	~60
5	Re-circulated 3274 bp fragment	Experimental	~60

B. Construction of 3' LuIII 3' *de novo* Strategy

Sample	DNA Added	Plate	Concentration of DNA (ng)
1	None	Control for Cell Growth	0
2	None	Control for Antibiotic	0
3	pUC19	Efficiency of Transformation Control	~20
4	pUC19/ BamHI/ CIAP	Control for Phosphatase	~30
5	pUC19/ BamHI/ CIAP + insert	Experimental	~50

Isolation of DNA Recombinants

The resultant plasmids from DH5α and SURE®2 transformed cells were purified by the alkaline lysis miniprep method, described by Ausubel *et al*. (5), and digested with various restriction enzymes. The restriction patterns for each possible clone are shown in Table 4. Large-Scale preparations were also made using the Qiagen Plasmid Midi Kit (QIAGEN, Inc., Santa Clarita, CA). DNA samples with a unique restriction pattern were sent to New Jersey Medical School for sequencing. The sequences were analyzed using the BLAST programs from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST), the ALIGN Program from the European Molecular Biology Network (www.ch.embnet.org) and the Seqweb Programs from the High Performance Computing Facilities (boreas.hpcf.upr.edu).

Table 4. Expected Restriction Patterns for each possible clone (bp)

Restriction Enzyme	Strategies used to construct the plasmid 3' LuIII 3'		
	Re-circulated 2998 bp fragment	Re-circulated 3274 bp fragment	*de novo* 3' LuIII 3'
*Eco*RI	2686, 312	2686, 588	3254
*Pme*I	2998	2998, 276	2978, 276
*Mlu*I	-	3274	3254
*Eco*RI/*Mlu*I	2998	2686, 294, 294	-
*Bam*HI	2998	3274	2686, 568
*Bam*HI/*Mlu*I	-	-	2686, 284, 284

(-) Not performed

Tissue Culture

HeLa (ATCC, Rockville, MD) cells were grown in Minimal Essential Medium (MEM Eagle) (MP Biomedicals, Aurora, OH) supplemented with 10% bovine calf serum (BCS) (HyClone, Logan, UT) and PSG (8mM Penicillin G, 3mM Streptomycin Sulfate, 200mM L-Glutamine). They were incubated at 37 ºC in 25 and/or 75 cm^2 plastic tissue culture flasks. For sub-culturing, the cells were rinsed twice with Phosphate-Buffered Saline (PBS) and incubated in 1X Trypsin (Difco, Detroit, MI) for 5 min at 37 ºC. Cells were harvested by centrifugation at 3500 rpm for 5 min at 4 ºC. The resultant pellet was resuspended in the medium described above and seeded into culture flasks at a proportion of 1:3.

Transfection Assay

HeLa cells were transfected by the DEAE-Dextran method and also by electroporation. Four different samples were required for each assay:

1. Negative control (flask 1)

2. LuIII infection (flask 2)

3. LuIII/3' LuIII 3' co-transfection (flask 3)

4. 3' LuIII 3' transfection (flask 4)

For the DEAE-Dextran method, the cells were grown to 70-80% confluency in four 25 cm^2 flasks. The medium was changed 2-4 hours before the initiation of the assay. The transfection mixture, consisting of 1.5 mL 1X MEM-10% BCS, 25 μL 80mM chloroqine, 30 μL 10 mg/mL DEAE-Dextran, and 5 μg 3' LuIII 3' DNA (for

flasks 3 and 4), was added to the cells and incubated for 4 hours at 37 ºC. The mixture was removed and 5 mL PBS-10% DMSO was added, and then incubated at room temperature for 4 min. The cells were rinsed twice with PBS and then incubated at 37 °C in 1X MEM-10% BCS.

After 24 h post-transfection, flasks 2 and 3 were infected with LuIII virus. Infection with LuIII was necessary to provide *in trans* the helper functions for the replication of 3' LuIII 3'. Cells were washed twice with PBS to eliminate the serum and then 500 μL of LuIII virus in 500 μL of 1X MEM was added to the flasks. The cells were incubated at 37 ºC for one hour. The mixture was removed and 1X MEM/10% BCS was added. The cells were incubated at 37 ºC until the low molecular weight DNAs were isolated at 96 h post-transfection, as described by Tam and Astell (72). DNA samples were rinsed, desiccated, and resuspended in 15 μL TE (10mM Tris-HCl, 1mM EDTA, pH 8.0).

To electroporate, HeLa cells were grown to 100 % confluency (4th-5th day after passage) in a 75 cm^2 flask. They were washed three times with PBS and then tripsinized at 37 ºC for 5 min. Cells were harvested by centrifugation at 3800 rpm for 7 min at 4 ºC. The pellet was resuspended in 10 mL PBS and centrifuged as described above. The pellet was resuspended in 7.2 mL 1X MEM and transferred into nine 1.5 mL tubes (800 μL each). Only four tubes were used for each assay. Approximately, 5 μg of pGLu883Δ*Xba* (57) (pGLu883 with deleted *Xba*I site at nt 7819) and 3' LuIII 3' were added to the corresponding tubes and incubated at 37 ºC for 10 min. The cells

were transferred to sterile cuvettes with a 4-mm gap width, and electroporated individually at 230 V and 960 μF using a capacitance discharge machine (Gene Pulser, Bio-Rad Laboratories, Hercules CA). After each pulse, 700 μL of MEM-10% BCS were added to the cuvette and the cells were resuspended carefully. The electroporated cells were incubated for 45 min at 37 ºC and then transferred to 25 cm^2 flasks containing 3 mL MEM-10% BCS. After an overnight incubation at 37 ºC, the medium was changed, and the cells were incubated at 37 °C until the low molecular weight DNAs were isolated at 96 h post-transfection, as described above.

Southern Blot Analysis

Samples were digested with *Dpn*I (G$_m$A/TC) (New England Biolabs, Beverly, MA) and *Mbo I* (/GATC) (New England Biolabs, Beverly, MA). The samples were electrophoresed on a 1.2% agarose gel in 1X TBE buffer at 80 V, and passively transferred onto a Zeta Probe nylon membrane (Bio-Rad Laboratories, Hercules, California) as described by Ausubel *et al.* (5). The LuIII genome sequence was used as a probe. Genomic clone pGLu883 was digested with *Bam*HI and the 5135 bp fragment corresponding to LuIII genome was isolated as described above and labeled by the random primed DNA labeling method with Digoxigenin-11-dUTP (Roche Applied Science, Indianapolis, IN). The blot was hybridized at 55 ºC for three hours and washed at 68 ºC. Detection was performed according to manufacturer's instructions (Roche Applied Science, Indianapolis, IN).

CHAPTER IV
Results and Discussion

The majority of the APVs contain non-identical hairpin structures at the 3' and 5' termini and encapsidate mostly minus strands. Parvovirus LuIII, has non-identical termini, nevertheless it encapsidates equal amounts of plus and minus strand, similar to AAV and B19. The terminal palindromes also display two alternative sequence orientations known as *flip* and *flop*. AAV shows *flip* or *flop* conformation with equal frequency at both ends of either DNA strand. MVM shows equally *flip* or *flop* at the right end, and exclusively the *flip* conformation at the left end. LuIII shows only *flip* at the left terminus of the minus strand and equal *flip* or *flop* at the right end of the plus and minus strands. The left end conformation of the LuIII-plus strand is still unknown. To explain the different patterns observed among parvoviruses, a kinetic hairpin transfer model has been proposed (16), in which the rates of hairpin transfer at the 3' termini define the encapsidation distributions of the different viruses.

A plasmid containing two copies of the LuIII-3' termini was constructed. The sequences were cloned into the *Eco*RI site of the pUC19 vector in the correct orientation (head to tail-tail to head). Nevertheless, this plasmid named 3' LuIII 3', has an insertion of unknown sequence between the two termini. The aim of this work was to eliminate the stuffer sequence of 3' LuIII 3' and then transfect the LuIII clone into human cells to compare its encapsidation pattern to that of the wild type genome.

Strategies followed to construct the clone 3' LuIII 3'

To construct a clone with only two copies of the left end, three different strategies were followed, as described under the Materials and Methods section. The possible recombinant molecules were initially propagated into *E. coli* DH5α cells. Due to the palindromic nature of the left hairpin sequence of the LuIII virus, another strain of *E. coli* named SURE®2 supercompetent cells was also chosen. Both strains are recombination (*recA1*) and endonuclease deficient (*endA1*), greatly increasing the insert stability and improving the quality of plasmid DNA prepared from mini-preps.

However, the SURE®2 supercompetent cells have deletions of *E. coli* genes involved in the UV repair system (*uvrC*) and the SOS repair pathway (*umuC*), helping in the cloning of irregular DNA structures with inverted repeats or secondary structures. SURE®2 supercompetent cells also have mutations in genes encoding the SbcB, RecJ and RecB proteins involved in recombination, preventing homologous recombination between the insert and host genome. Both types of cells were transformed using calcium chloride and heat shock at 42 °C, as described by Ausubel *et a.l* (5). The transformation efficiency expected by this protocol for the *E. coli* DH5α cells ranged from 1 x 10^6 to 1 x 10^8 transformants/μg DNA. The transformation efficiency expected for the SURE®2 supercompetent cells is 1 x 10^9 transformants/μg DNA.

A.   Elimination of the stuffer sequence between the two 3' termini

The plasmid 3' LuIII 3' was digested independently with *Pme*I or *Mlu*I. *Pme*I cuts 3' LuIII 3' at nt 140 of the LuIII sequence, generating two fragments of ~576 and 2998 bp. *Mlu*I cuts the minigenome at nt 278 of the LuIII sequence, generating two fragments of ~300 and 3274 bp. DNA electrophoresis confirmed the presence of the expected fragments (Figure 8 and Figure 9). The fragments of 2998 (from the *Pme*I digestion) and 3274 bp (from the *Mlu*I digestion), corresponding to the pUC19 sequence containing two copies of the LuIII-3' termini at each side of the vector, were isolated from the agarose gel as described previously.

To confirm the nature of the desired fragments, 5% of each purified product was electrophoresed (Figure 10). Once confirmed, the fragments were ligated independently using the T4 DNA ligase. The possible recombinant molecules were transformed into *E. coli* DH5α or SURE[®]2 super-competent cells.

A total 18 transformations of 15 different ligation reactions were done with the re-circularized fragment of 2998 bp, and 10 transformations of 10 different ligation reactions were done into *E. coli* DH5α with the re-circularized fragment of 3274 bp, and (Table 5). A total of 8 transformations of 8 different ligation reactions were done into SURE[®]2 supercompetent cells with each 2998 and 3274 bp re-circularized fragments (Table 5). The transformation efficiency of *E. coli* DH5α cells was ~1.4 x 10^6 CFU/μg DNA. The transformation efficiency of SURE[®]2 cells was ~1 x 10^6, a

value below that expected, this probably due to manipulation during the process of
making them competent.

Table 5. Transformations of *E. coli* DH5α and SURE®2 supercompetent cells

Transformation (~15 ng DNA[a])	Number of colonies						Total
	DH5α			SURE®2			
	*Pme*I	*Mlu*I	*de novo*	*Pme*I	*Mlu*I	*de novo*	
1	3	0	9	0	0	0	**12**
2	17	0	14	2	0	0	**33**
3	0	0	12	10	0	0	**22**
4	0	0	14	6	2	6	**28**
5	0	3	15	2	0	1	**21**
6	4	0	9	10	3	9	**35**
7	0	6	8	0	7	0	**21**
8	0	0	8	0	0	0	**8**
9	0	0	-	-	-	-	**0**
10	0	0	-	-	-	-	**0**
11	0	-	-	-	-	-	**0**
12	12	-	-	-	-	-	**12**
13	19	-	-	-	-	-	**19**
14	0	-	-	-	-	-	**0**
15	3	-	-	-	-	-	**3**
16	3	-	-	-	-	-	**3**
17	6	-	-	-	-	-	**6**
18	0	-	-	-	-	-	**0**
Total	**67**	**9**	**89**	**30**	**12**	**16**	**223**

[a] Estimated concentration is based on the amount of vector in the ligation reaction

The control plates used in the transformation assay resulted as follows. Normal growth of non-transformed cells was observed on the plates without ampicillin, demonstrating that the cells were viable after the heat shock. No growth of non-transformed cells was observed on the plates containing ampicillin demonstrating that the cells were sensitive to the antibiotic and that the cells were not contaminated. The original construct 3' LuIII 3' has two copies of the left end termini cloned into the *Eco*RI site of the multiple cloning site (MCS) in the pUC19 vector. Since the MCS is inserted after the fourth codon of the *lacZα* gene, the LuIII insert interrupts the expression of the gene, and hence only white colonies grew on the selective media.

A total of approximately 76 and 42 minipreparations from DH5α and SURE®2 supercompetent cells, respectively, were made (Table 5). The samples were digested with *Eco*RI and analyzed by DNA electrophoresis. If the clone was a result of the re-circularized 2998 bp fragment, the *Eco*RI digestion should generates two fragments of 312 and 2686 bp, corresponding to the LuIII-3' hairpin sequences (1-140,140-1) and the vector pUC19, respectively (See table 4). If the construct was a result of the re-circularized 3274 bp fragment, the *Eco*RI digestion would generate two fragments of 588 and 2686 bp, corresponding to the LuIII-3' hairpin sequences (1-278, 278-1) and the vector pUC19, respectively. However, none of the DNA samples analyzed exhibited the restriction patterns described above.

The majority of the samples obtained from the re-circularized 3274 bp fragment, resulted in only one band of ~3000 bp (Figure 16). The majority of the samples obtained from the re-circularized 2998 bp fragment were resistant to *Eco*RI digestion and migrated similar to the uncut sample of pUC19. The samples were not digested with other restriction enzymes.

Figure 16. Digestions with *Eco*RI. Lanes 2-9: *Eco*RI digestions of several recombinant molecules obtained from the re-circularized 3274 bp fragment. The digestions were electrophoresed on a 1% agarose gel in 1X TBE buffer at 80 V. Sizes of the 1Kb DNA Ladder (lane 1) (Gibco, Gaithersburg, MD) are indicated.
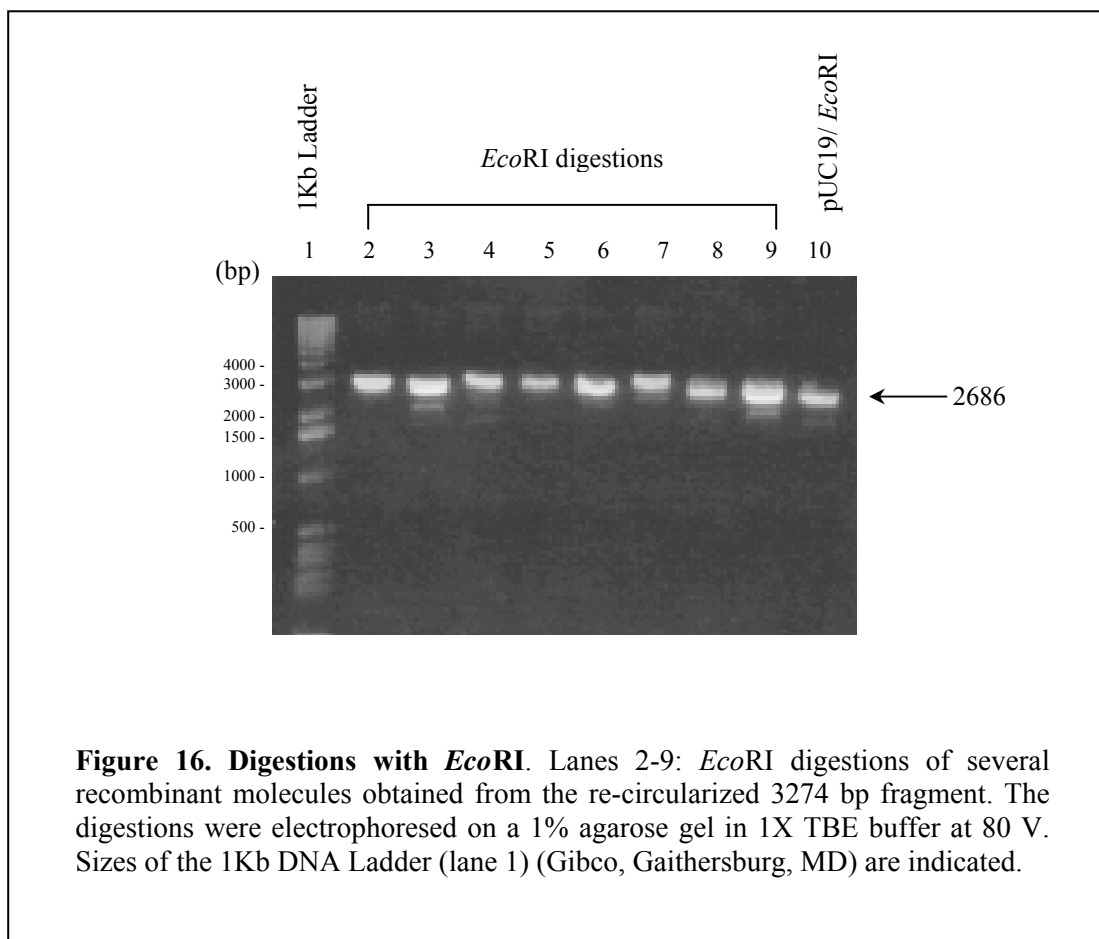
Many investigators have confirmed that the cloning of parvovirus termini is a difficult task, due to the nature of their palindromic sequences and their secondary structures. Sequences in the left end hairpin have proven to be difficult to manipulate due to their palindromic nature. Cotmore and Tattersall (28) reported that the palindromic inserts showed a greater tendency for deletions even in some recombination-deficient strains of *E. coli*, probably due to the complex structures assumed by the inserts. Liu *et al.* (55) also reported inherent difficulties in cloning hairpins, resulting in many incorrect and presumably rearranged clones. An intramolecular slipped mispairing mechanism has been implicated in the generation of deleted variants of the parvovirus genomes (73).

Taking this into account, it is then possible that the fragments of approximately 3000 bp that were generated when the plasmids were digested with *Eco*RI, could represent the pUC19 vector with an insert of one copy of the LuIII hairpin. The other hairpin was deleted likely due to an intramolecular recombination. The molecules that were resistant to *Eco*RI digestion could represent plasmids in which the whole insert has been deleted including the *Eco*RI site of the pUC19 vector. The LuIII insert could be forming a complex hairpin structure *in vivo* possibly due to its palindromic nature that is removed by slipped mispairing during replication. Difficulties in the sequencing of the termini region could support this observations.

B. Construction of the recombinant molecule 3' LuIII 3' *de novo*

An attempt to construct the plasmid 3' LuIII' 3 *de novo* was made. To isolate the left end hairpin sequence of the LuIII virus, the genomic clone pGlu883 was double digested with *Bam*HI/*Mlu*I. This digestion generated three fragments of approximately 284, 2686, and 4861 bp. The 284 bp fragment contains the left end sequence of LuIII (nts 1-278), the 2686 bp fragment corresponds to the pUC19 vector sequence, and the 4861 bp fragments contains nts 279-5135 of the LuIII sequence (Figure 13) The 284 bp fragments containing the left end hairpin sequences were successfully isolated from the agarose gel as described previously (Figure 14) and ligated to each other using the T4 DNA ligase.

Since numerous combinations of the ligated 284 bp fragments could be formed, the ligation was digested with *Bam*HI, generating fragments of 568 bp, corresponding to two copies of the left end hairpin (1-278, 278-1) that were ligated through the *Mlu*I site. The *Bam*HI digestion was electrophoresed and the 568 bp fragment was isolated from the agarose gel (Figure 15). Concurrently, pUC19 vector DNA was digested with *Bam*HI, and treated with CIAP, which removes the phosphate group at the 5' end of the nick site. This treatment inhibits the formation of a phosphodiester linkage between the 3' and 5' end of the linear vector, favoring the cloning of the 568 bp insert. The purified 568 bp fragment was ligated into the *Bam*HI site of pUC19 using T4 DNA ligase, and the possible recombinant molecules were transformed into *E. coli* DH5α or SURE[®]2 supercompetent cells.

A total of 8 transformations of 7 different ligation reactions were made into each *E. coli* DH5α and SURE®2 supercompetent cells (Table5). The transformation efficiency of *E. coli* DH5α cells was ~3.5 x 10^6 CFU/μg DNA. The transformation efficiency of SURE®2 was ~9.2 x 10^5 CFU/μg DNA, a value lower than expected. The results of the control plates for cell growth and the antibiotic were identical to those described above. Normal growth of non-transformed cells was observed on the plates without ampicillin, and no growth was observed on the plates containing ampicillin, demonstrating respectively, that the cells were both viable and sensitive to the antibiotic. No growth was observed on the control plate for the phosphatase, suggesting that the pUC19 was not ligated, and thus the cells were ampicillin susceptible. White colonies grew on the experimental plates suggesting that the recombinant molecules might contain the LuIII insert.

Approximately, 16 and 89 minipreparations from SURE®2 and DH5α cells, respectively, were made (Table 5) and analyzed by digestion with *Bam*HI, which separates the LuIII insert from the pUC19 vector. DNA electrophoresis of the *Bam*HI digestions suggested that six recombinant molecules obtained from *E. coli* DH5α cells could contain the expected insert. They were arbitrary named mp4, mp6, mp20, mp29, mp38 and mp39. For a better analysis, large-scale preparations of these samples were made using the QIAGEN Plasmid Midi Kit, and subsequently digested with different restriction enzymes.

Characterization of the six DNA Recombinants obtained from *E. coli* DH5α cells

The first five preparations were characterized by digestion with *Bam*HI, *Mlu*I, *Pme*I and *Bam*HI/*Mlu*I (Figure 17 and Figure 18). If the construct is well oriented and contains two copies of the LuIII-left hairpin, digestion with *Bam*HI should produce two fragments of 568 and 2686 bp corresponding to the LuIII insert and the pUC19 sequence, respectively. Digestion with *Mlu*I should linearized the plasmid generating one fragment of 3254 bp. *Pme*I should cut twice in the LuIII insert generating two fragments of 276 and 2978 bp. Digestion with *Bam*HI/*Mlu*I should produce two fragments of 284 and 2686 bp, corresponding to the two copies of the LuIII-3' end and the pUC19 sequence, respectively. However, none of the analyzed plasmids exhibited the expected restriction pattern, as described above.

The recombinant molecule mp4 digested with *Bam*HI generated two fragments of 618 and 2686 bp; digestion with *Bam*HI/*Mlu*I resulted in three fragments of 284, 334 and 2686 bp. The sample mp6 digested with *Bam*HI generated two fragments of 650 and 2686 bp; digestion with *Bam*HI/*Mlu*I generated three fragments of 284, 366 and 2686 bp. The sample mp20 digested with *Bam*HI, exhibited two fragments of 583 and 2686 bp; digestion with *Bam*HI/*Mlu*I generated three fragments of 284, 299 and 2686 bp. The sample mp29 digested with *Bam*HI exhibited two fragments of 628 and 2686 bp; digestion with *Bam*HI/*Mlu*I generated three fragments of 284, 344 and 2682 bp. The sample mp38 digested with *Bam*HI generated two fragments of 543 and 2686 bp; digestion with *Bam*HI/*Mlu*I generated three fragments of 259, 284 and 2686 bp. All the samples were digested independently with *Mlu*I and *Pme*I. Both digestions

exhibited one fragment of approximately 3300 bp (Data not shown). This restriction analysis suggested that all five plasmids have a single copy of the left hairpin of LuIII ligated to a sequence of ~300 bp, similar to the size of the hairpin.

The mp39 was first characterized by digestion with *Bam*HI/*Mlu*I, which generated three fragments of ~250, 284 and 2686 bp (Figure 19A). Digestions with *Pme*I, *Mlu*I, *Bam*HI, and *Pst*I, generated a restriction pattern very similar to that observed for the construct 3' LuIII 3' (Figure 19B). Digestion with *Bam*HI generated two fragments of ~800 and 2686 bp. The *Pme*I digestion generated two fragments of ~500 and 2978 bp. The *Mlu*I digestion generated two fragments of ~250 and 3254 bp. To determine the size of the plasmid, mp39 was digested with *Pst*I, which was expected to cut only once in the pUC19 vector, generating one fragment of ~3500 bp.

The restriction analysis suggests that the plasmid mp39 contains two copies of the LuIII-left end hairpin (nts 1-278) cloned in the correct orientation into the *Bam*HI site of the vector pUC19, but with a stuffer sequence of almost 250 bp, ~50 bp smaller than the stuffer sequence of the plasmid 3' LuIII 3'. The possible explanations for the presence of a stuffer sequence between the two termini in both constructs 3' LuIII 3' and mp39 are unknown. It was shown several times that two copies of the left end termini were successfully ligated *in vitro* (Figure 14). This suggests that the insertion of the stuffer sequence occurs inside the bacteria, possibly during plasmid replication. The observations suggest that a clone containing two hairpin copies of LuIII would not be obtained without a stuffer sequence, probably due to a steric impediment.

A.

B.
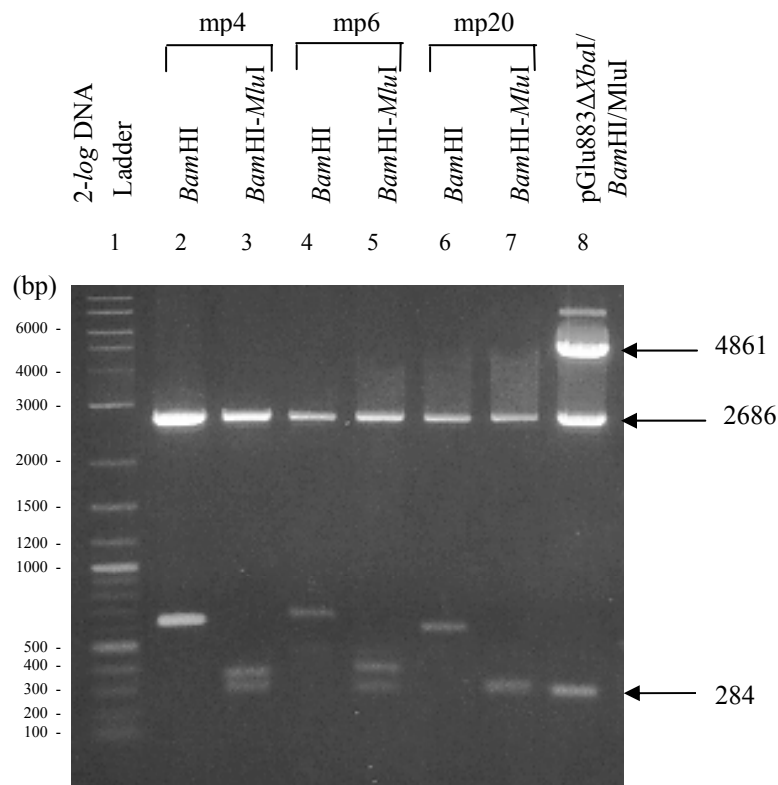
Restriction Enzyme	Expected Pattern for 3' LuIII 3'	mp4	mp6	mp20
*Bam*HI	568	618	650	583
	2686	2686	2686	2686
*Bam*HI/*Mlu*I	284	284	284	284
	284	334	366	299
	2686	2686	2686	2686

Figure 17. Digestions of clones mp4, mp6 and mp20 with *Bam*HI and *Bam*HI/*Mlu*I. (A) The digestions were compared to the *Bam*HI/*Mlu*I digestion of pGLu883Δ*Xba*. The samples were run on a 1.2% agarose gel in TBE buffer at 76V. Sizes of the 2-*log* 1Kb DNA Ladder (lane 1) (New England Biolabs, Beverly, MA) are indicated. (B) Sizes of the fragments generated for each plasmid are indicated.

A.

B.
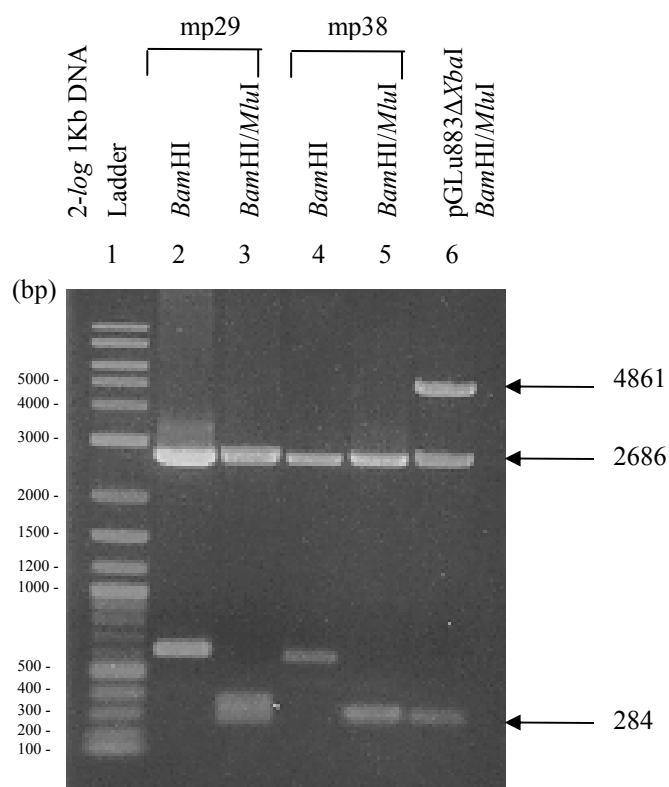
Restriction Enzyme	Expected Pattern for 3' LuIII 3'	mp29	mp38
*Bam*HI	568 2686	628 2686	543 2686
*Bam*HI/*Mlu*I	284 284 2686	284 344 2686	259 284 2686

Figure 18. Digestions of clones mp29 and mp38 with *Bam*HI and *Bam*HI/ *Mlu*I.
(A) The digestions were compared to the *Bam*HI/*Mlu*I digestion of pGLu883Δ*Xba*.
The samples were run on a 1.2% agarose gel in TBE buffer at 76V. Sizes of the 2-*log* 1Kb DNA Ladder (lane 1) (New England Biolabs, Beverly, MA) are indicated.
(B) Sizes of the fragments generated for each plasmid are indicated.

A.

B.
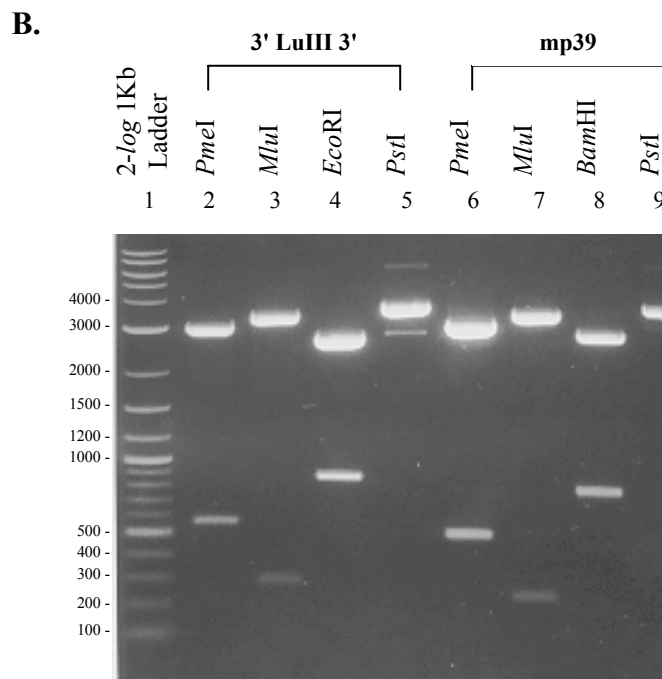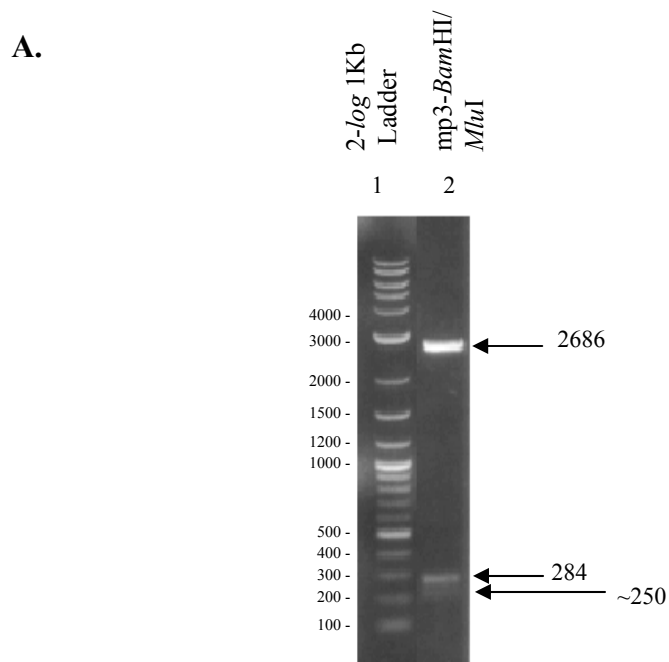
Figure 19. **Digestions of 3' LuIII 3' and mp39.** (A) *Bam*HI/ *Mlu*I digestion of mp39. (B) Lanes 2-5: Digestions of 3' LuIII 3'. Lanes 6-9: Digestions of mp3. The samples were run on a 1.2% agarose gel in TBE buffer at 73 V. Sizes of the 2-*log* 1Kb DNA Ladder (lane 1A, 1B) (New England Biolabs, Beverly, MA) are indicated.

DNA Sequencing of mp4, mp6, mp20, mp29 and mp38

To confirm the nature of the inserted sequences, the DNA samples were sequenced by the Sanger ddNTP Chain Termination Sequencing method using Universal Forward (M13F) and Reverse (M13R) primers at New Jersey Medical School. The sequences confirmed the presence of LuIII nts 1 to 278 ligated to another sequence of nearly the same size. The insertions differ considerably in size (259-366 nts) and sequence, and conserved the restriction sites for *Bam*HI and *Mlu*I. The results also revealed that all the clones share in common the same orientation in pUC19 with respect to the Reverse and Forward Primers, conserving the LuIII sequence at the 5' end and the unknown sequence at the 3' end (Figure 20). The sequencing reaction with the Reverse primer was not possible, likely due to the highly palindromic nature of the terminus.

Figure 20. Generalized map of the *E. coli* DH5α -LuIII recombinant plasmids obtained containing the genomic sequence.
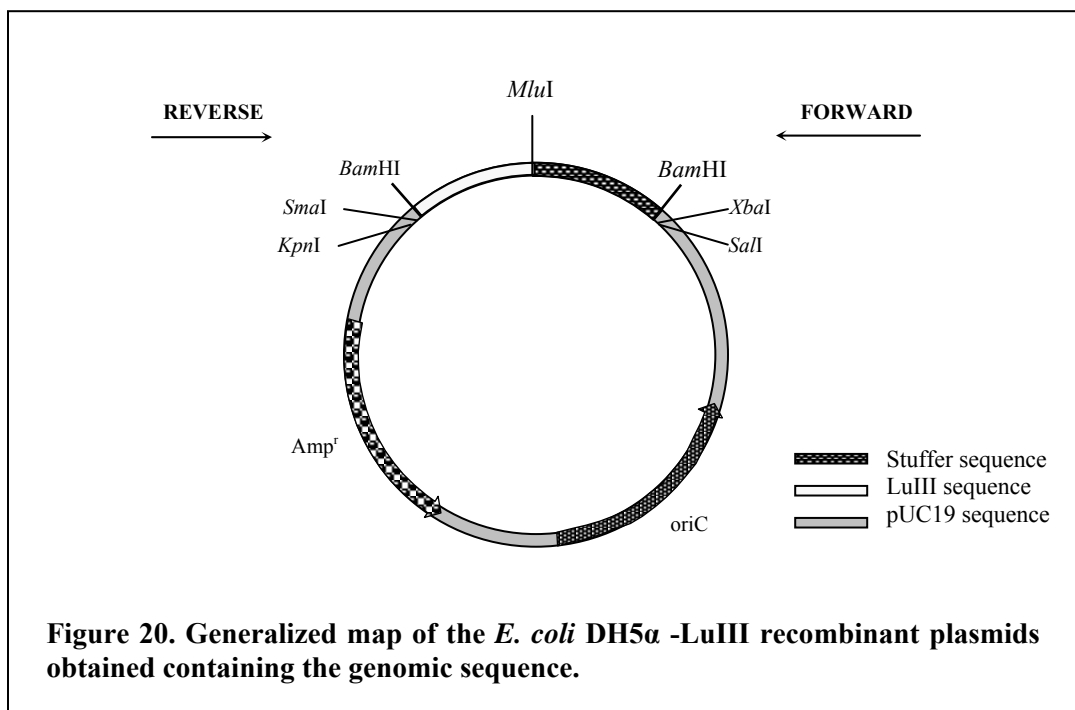
Analysis in BLAST, LALIGN and MFold Programs

To identify the origin of the inserted sequence and a possible relationship with the left end palindrome of the LuIII virus, the peculiar sequences were analyzed using computerized programs of Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI). According to BLAST, each sequence has a 100% homology with sequences of the *E. coli* K12 genome, each encoding different genes. The restriction sites for *Bam*HI and *Mlu*I were identified in the sequences analyzed. Table 6 summarizes the results that were obtained from the BLAST program.

Table 6. Characterization of the inserted sequences according to BLAST Program

Sample	Size of the *E. coli* insertion (bp)	Gene	Synonym	Function
mp4	334	*yehV*	b2127, mlrA	Putative transcriptional regulator
mp6	366	*rpmC*	b3312	50S Ribosomal subunit protein L29
mp20	299	*yrfI,*	b3401	Redox regulated molecular chaperone
		yhgE	b3402	Subcelullar putative transport
mp29	344	*sfhB*	b2594	Suppressor of ftsH mutation
mp38	259	*lexA*	b4043	Regulatory gene for SOS (lexA) regulon

To study the possibility that a recombination event between the *E. coli* genome and the LuIII sequence occurred, the insertions were analyzed by sequence alignment using the LALIGN Program of the European Molecular Biology Network (EMBnet). Both local and global alignments were made. The global alignments between the LuIII

and the *E.coli* sequences showed an overall identity of 44-48%. Interestingly, the local alignments showed that all five *E. coli* sequences share in common a 55-67% sequence identity with the region containing nts 110-190 of the LuIII DNA, just at the end of the LuIII-palindromic termini. Table 7 shows some of the major identities within this region. The position of the overlapping sequence varies throughout the *E. coli* sequences.

Table 7. Local alignments between the LuIII and the *E. coli* inserted sequences using the ALIGN program from the EMBnet

Sample	Overlapped Sequence	Identity (%)	Score (bits)	Expected Value
mp4	``` 110 120 130 140 LuIII AGTTCTAAAAATGATAAGCGGTTCAGGGAGTTTA ::::: :: :: : ::: :: :::: : : :: mp4 AGTTCAAAGAAAGTTAAACG---CAGGCAATGTA 300 310 320 330 ```	67.6	61	1e+03
mp6	``` 120 130 140 150 LuIII TGATAAGCG-GTTCAGGGAGTTTAA-ACCAAGGCGCGAAA ::: ::: : ::: : : :: : :: ::: :: :: :: mp6 TGAGAAGAGCGTTGAAG-AGCTGAACACCGAGCTGCTGAA 240 250 260 270 ```	65.0	44	9.6e+03
mp20	``` 120 130 140 150 LuIII AAAAATGA-TAAGCGGTTC-AGGGAGTTTAAACCAAGGCGCGAA :::::: : : : : : : ::: : ::: : :: :::: mp20 AAAAATTACTCACAGCTGCGAAGGA-TGGAAAACTGGGGACGAA 110 120 130 140 ```	61.4	37	1e+04
mp29	``` 130 140 150 160 LuIII GGTTCAGGG---AGTTTAAACCAAGGCGC--GAAAAGGAAGTGGGCG :::: : :: ::::: ::: :: ::::: : : :::: : mp29 GGTTAACGGCAAAGTTTGTGATAAGCCGAAAGAAAAAGTATTGGGTG 20 30 40 50 60 ```	61.7	53	4.1e+03
mp38	``` 140 150 160 LuIII TTTAAACCAAGGCGCGAAAAGGAA : :: ::: ::: : :: ::: mp38 TATACACCCAGGGGGCGGAATGAA 170 180 ```	62.5	39	1e+04

To study the possibility that the inserted sequences might assume secondary structures such as that of the left hairpin of LuIII, the sequences were analyzed in the MFold Program of SeqWeb. The program showed very stable complex structures with a ΔG° of at least −93 kcal/mol indicating that all the five reactions are exergonic and therefore spontaneous (Figure 21). However, very similar results were also obtained when random sequences of *E. coli* K12 and of human origin were analyzed, suggesting that there is no relationship between the secondary structures of the *E. coli* sequences and the left hairpin of LuIII (Figure 22).

The analyses suggest that *E. coli* genomic DNA was present in the starting materials used for cloning the left end hairpin of LuIII virus. The starting materials were obtained from *E. coli* preparations using the QIAGEN Plasmid Midi Kit. The separation of plasmids from chromosomal DNA is based on the co-precipitation of the chromosomal DNA with insoluble complexes containing salt, detergent, and protein. The plasmid DNA should remains on the clear supernatant. However, vigorous treatment during the lysis procedure will share the bacterial chromosome, leaving free chromosomal DNA fragments in the supernatant. Since the chromosomal fragments are chemically indistinguishable from the plasmid DNA under the conditions used, the two species will be elute under the same salt conditions. Assuming that the preparation of pGLu883 was contaminated, the DNA plasmids as well as the genomic DNA were double digested with *Bam*HI and *Mlu*I. Genomic DNA fragments of approximately 300 bp were probably isolated from the agarose gel when isolating the 284 bp fragment of pGLu883, and were ligated with equal frequency during the ligation step.
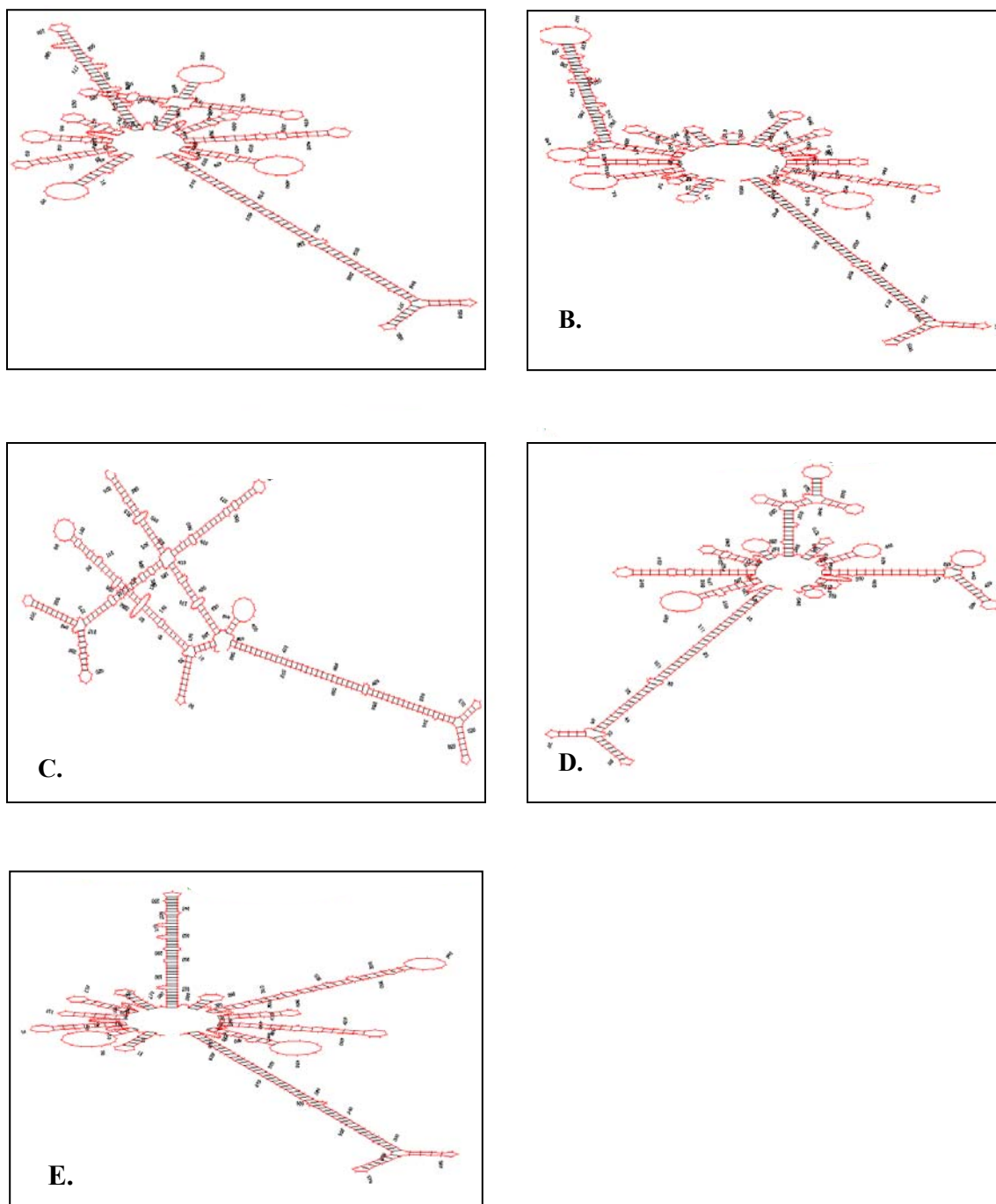
Figure 21. Squiggle plot of the inserted sequences in mp4, mp6, mp20 and mp38.
(A) mp4, ΔG° -115.1 kcal/mol; (B) mp6, ΔG° -125.2 kcal/mol; (C) mp20, ΔG° -109.7 kcal/mol; (D) mp29, ΔG° -101.5 kcal/mol; (E) mp38, ΔG° -127.6 kcal/mol.
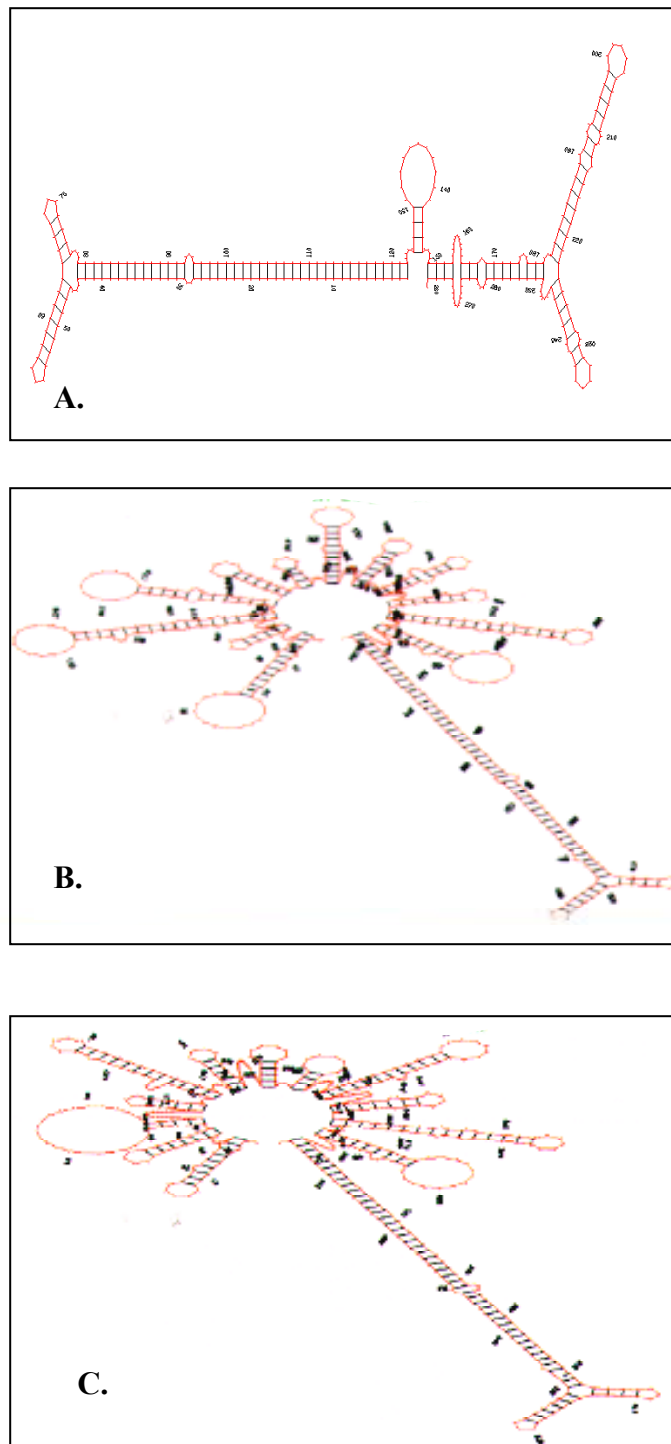
Figure 22. Squiggle plot of the *E. coli* and human sequences randomly selected.
(A) Secondary structure of nts 1-273 of the LuIII virus, ΔG° -71.6 kcal/mol; (B) Random sequence of the *E. coli* genome, ΔG° -106.5 kcal/mol; (C) Random sequence of human origin, ΔG° -101.5 kcal/mol.

Transfections of 3'LuIII 3' and Southern Blot Analysis

To study the possible replication of 3' LuIII 3' in eukaryotic cells, the construct was co-transfected with pGLu883Δ*Xba*, the genomic clone of LuIII that provides the necessary *trans* acting factors for replication of the minigenome. It has been suggested that the non-structural protein NS1 makes the excision of the APVs viral genomes from infectious molecular clones, by introducing a single-stranded nick possibly at the 5' end of the viral genome. If the minigenome replicates under these conditions, it is thus obligated to contain all the *cis*-acting sequences required for excision and DNA replication.

The plasmids were co-transfected into HeLa cells by DEAE-Dextran method and also by electroporation. A total of 16 transfections were performed: 9 by the DEAE-Dextran method and 7 by electroporation. To determine if the plasmids pGLu883Δ*Xba* and 3' LuIII 3' had replicated in eukaryotic cells, the samples were digested with *DpnI* and *Mbo*I. *DpnI* digests only methylated DNA, whereas *MboI* recognizes the same sequence but will only digest un-methylated DNA. This feature allows differentiation between input and output DNA. A Southern blot analysis of a transfection by electroporation is shown in Figure 23. The blot was hybridized with the LuIII full-length genome, labeled by random primed Digoxigenin-11-dUTP, and exposed for 16 H. The samples from this transfection were not digested with *Dpn*I.
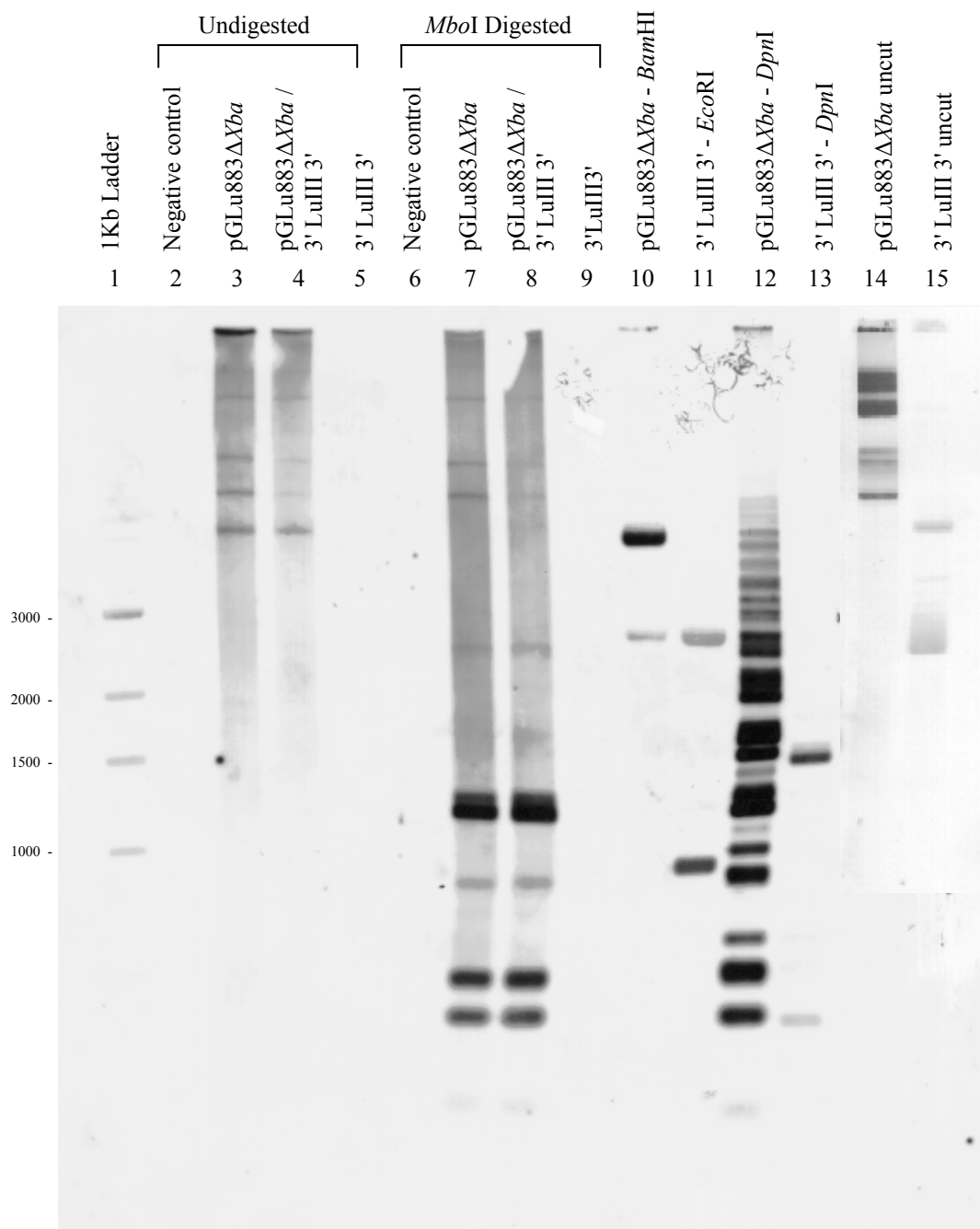
Figure 18. Southern blot analysis of a transfection by electroporation. Lanes 2-5 and 6-9 show the samples undigested and digested with *Mbo*I, respectively. Lanes 10-15 show DNA samples used as controls. The samples were run a 1.2% agarose gel in 1X TBE buffer at 80 V. The blot was hybridized at 55 ºC and probed with the *Bam*HI insert of pGLu883Δ*Xba*. The radiograph shown was exposed for 16 H. Sizes of the 1Kb DNA Ladder (lane 1) (New England Biolabs, Beverly, MA) are indicated.

To determine the nature and the sizes of the possible fragments, several DNA samples were run as controls. To identify the LuIII double-stranded DNA, pGLu883Δ*Xba* was digested with *Bam*HI (lane 10), which generates two bands of 2686 and 5145 bp corresponding to the pUC19 and LuIII sequence, respectively. To identify the replicated fragments of the LuIII virus in the transfected samples with pGLu883Δ*Xba* after *Mbo*I digestion, the plasmid pGLu883Δ*Xba* was digested with *Dpn*I (lane 12). The hybridized fragments that are common in both samples will correspond to LuIII sequences. If the LuIII sequence in the plasmid 3' LuIII 3' can be excised and replicated when co-transfected with pGLu883Δ*Xba*, a band of ~900 bp will be detected. This fragment does not contain restriction sites for *Dpn*I and thus for *Mbo*I. To identify this band, the plasmid 3' LuIII 3' was digested with *Eco*RI (lane 11), generating two bands of ~900 and 2686 bp, corresponding to LuIII and pUC19 sequences, respectively. To recognize the possible bands of the input DNA in the transfected samples, uncut samples of pGLu883Δ*Xba* and 3' LuIII 3' were also electrophoresed (lanes 14 and 15, respectively).

Lanes 2-5 show the samples undigested. Lane 2 represents the negative control, in which no DNA was added. No bands were observed in this lane indicating that no non-specific binding occurred between the HeLa genomic DNA and the LuIII probe. Lanes 3 and 4, representing the samples of the transfection of pGLu883Δ*Xba* and the co-transfection of 3' LuIII 3' with pGLu883Δ*Xba*, respectively, show several bands that vary from ~5 to ~28 kb. Lane 5, representing the transfection of 3' LuIII 3' alone shows no bands.

Lanes 6-9 show the samples digested with *Mbo*I. Lane 6, representing the negative control, shows no bands. Lane 7 and 8, which represent the transfection of pGLu883ΔX*ba*, and the co-transfection of 3' LuIII 3' with pGLu883ΔX*ba*, respectively, exhibit the same pattern of bands. The upper bands were *Mbo*I resistant, indicating that they correspond to methylated input DNA and these could represent concatamers of the input plasmid pGLu883Δ*Xba*. These bands were also identified in lane 3, 4, and 14. The lane 14 corresponds to uncut plasmids of pGLu883Δ*Xba*.

The band of ~5 kb observed in lane 3 and 4 now disappears on lane 7 and 8, showing that it was *Mbo*I susceptible, and therefore is a result of LuIII replication. It corresponds to LuIII double-stranded DNA. Since LuIII virus generates equal amounts of plus and minus strand, these reanneal to each other and migrate as a double stranded molecule. This can also be confirmed by comparison with the sample in lane 10 that shows pGLu883Δ*Xba* digested with *Bam*HI. The lower bands in lanes 7 and 8 were generated by *Mbo*I digestion. The pattern of bands is identical to that observed in lane 9, which correspond to partial *Dpn*I digestion of pGLu883Δ*Xba* (lane 13), thus they correspond to LuIII sequences. Lane 9, representing the transfection of 3' LuIII 3' alone, shows no bands.

If replication of 3' LuIII 3' occurs, a band migrating at approximately 900 bp will be detected in both undigested and digested samples with *Mbo*I, since the insertion does not have any restriction sites for *Dpn*I nor *Mbo*I. This band will only appear in the presence of the helper plasmid, pGLu883Δ*Xba*. Bands of approximately

the half size of the ~900 bp fragment were also expected, that would correspond to ssDNA. However, no bands of ~900 bp or half their sizes were observed in the co-transfected samples (lanes 4 and 8). These results suggest that 3' LuIII 3' was not capable of excision and/ or replication when pGLu883Δ*Xba* was provided *in trans*. Some reasons that could explain or justify the results obtained are discussed below.

Studies of defective particles of AAV and MVM indicate that particles as small as 15% of the size of the wild type genome are able of replicating. The defective particles are generated spontaneously during high-multiplicity infections and always retain the two terminal palindromes (22). Defectives particles derived from the autonomous parvovirus MVM, have shown that the smallest sequence retained of the left terminus ends at nt 256 and comprises the 3' palindrome and the P4 promoter (22). This data suggests that for 3' LuIII 3' the size of the insert should not have been the reason for the lack of evidence of replication.

Parvovirus DNA replication starts when the 3' hydroxyl group at the left end of the viral genome primes the synthesis of a complementary strand, leading the formation of a double stranded replicative form known as the cRF. I*n vitro* studies have shown that the cRF of autonomous parvovirus like MVM terminates in closed hairpins at both ends, making cRF a major, or even obligatory intermediate of parvovirus replication (6), but only the right-end hairpin is resolved in the presence of NS1 (6, 76). The cRF is re-opened and copied, producing a right end extended form (5' eRF) followed by unfolding of the hairpin and coping of the terminal sequence.

This leads to the formation of dimeric RF (dRF) and higher-order concatamers molecules that would be resolved into monomeric (mRF) RF DNA. If the wild type LuIII virus behaves similar to MVM and forms the cRF, the replication of two copies of the left end such as in 3' LuIII 3' is expected to result in a dead molecule that could not be resolved by NS1.

Although the terminal palindromic sequences are essential for the replication of the APVs genome, the right and left terminal sequences are not equivalent in function (72). Different biochemical reactions occur at the left and right terminus during replication. The sequences at the right terminus may contain stronger initiation signals than those at the left end (72, 73). According to the modified rolling hairpin model of MVM replication, the right end origin is active in the covalently closed hairpin configuration and also in the extended right end telomere (34, 76). The latter observation could explain the proposal that NS1 is responsible for the excision of the viral genomes from infectious molecular clones. In contrast, the MVM left end inverted repeat does not constitute a replication origin in the hairpin configuration and needs to be copied in the form of a left-to-left end bridge to be subsequently resolved at the multimeric RF DNA stage (3, 6, 25, 34, 55).

When the dimmer bridge origin is compared with the left end viral sequence in parvovirus DNA clones, it becomes apparent that the left terminus is an incomplete origin of replication. A competent replication origin contains, among other things an NS1 nick site. The nicking reaction has been involved in the excision of parvovirus

DNA from recombinant plasmids. It has been shown that the excision and subsequent replication of H1 and MVM virus genomes from infectious molecular clones were greatly enhance when a consensus NS1 resolution site was reconstituted at the junction between the left hand viral terminus and the plasmid DNA (47). If like MVM, the left end terminus of LuIII is only processed when present as a bridge in the dimer RF but not as a hairpin in monomeric replicative form, either of the left end termini in 3' LuIII 3' would be recognized by NS1. As a result, the LuIII insert would not be excised from the plasmid pUC19, and hence no replication is expected to occur.

MVM minigenomes containing either two right (RR) or two left termini (LL) were shown to be replication competent suggesting that both termini contain the genetic information necessary for the excision and initiation of DNA replication (72). The pLL was constructed by substituting the MVM right end hairpin sequence for the left end hairpin sequence, and its replication was shown to be less efficient than the pRR (72, 73). Additional studies suggested that sequences within the region of nts 4489 to 4695, inboard of the 5' palindrome are required for efficient DNA synthesis (72, 73); gross deletions within this region, called the Internal Replication Sequence or IRS, were shown to reduce or abolish the replication of MVM minigenomes (72). Insertions of the IRS into defective minigenomes were able to activate DNA replication (73). These results were extended until identifying three specific short sequence elements, which contribute to the activity of the IRS region (12). It was suggested that these elements appear to form a multipartite origin of DNA replication similar to that observed in simple eukaryotic origins, such as the ARS1 of

Saccharomyces cerevisiae. It is then possible that the clone 3' LuIII 3' could need other *cis* acting sequences for its replication, as those present in the MVM pLL genome.

The mechanism by which LuIII encapsidates either strand of its genome with equal frequency remains unknown. Dependovirus AAV, which encapsidates equal amounts of plus and minus strand, replicates by a hairpin transfer reaction that involves DNA strand-specific cleavage and displacement synthesis. This type of resolution takes place at both ends of double stranded DNA intermediates, and is driven by the AAV nonstructural proteins Rep68 and Rep78. In contrast to MVM, the hairpin structure of AAV was found to be a preferred target for Rep binding and subsequent site-specific nickase (67). This phenomenon was not observed in LuIII. The evidence suggests that LuIII virus replication is different from that of and AAV and MVM. Another possibility is that the properties of the non-structural proteins of each virus differ among the species. The possibility that inadvertently, the left end sequences in 3' LuIII 3' have been disrupted still remains.

CHAPTER V

Conclusion

Three different attempts were performed to obtain a clone consisting of two copies of LuIII-3' termini cloned into the plasmid vector pUC19; irrespective of the strategy used, a 3' LuIII 3' clone without a stuffer sequence was not obtained. Only one construct with a smaller stuffer sequence between the two termini was obtained. It was arbitrary named mp39. The plasmid 3' LuIII 3' was co-transfected with pGLu883Δ*Xba* into HeLa cells by two different approaches: the DEAE Dextran method and electroporation. When the plasmids were co-transfected, only pGLu883Δ*Xba* was shown to replicate. The results suggest that 3' LuIII 3' was not capable of replicating when the helper functions were provided *in trans*.

CHAPTER VI

Recommendations

The following recommendations should be considered in order to continue with the initial aim of this work.

1. Confirm by sequencing the nature of the sequence of the LuIII-left ends in 3' LuIII 3' and in mp39.

2. Construct a molecule containing two left end termini flanking the full length genome; there with providing functions in *cis*.

3. Co-transfect the plasmids 3' LuIII 3' or mp39 with the MVMi infectious clone, to determine if the resolution of the termini are dependant on the nonstructural protein NS1.

4. To study the nature of the strands(s) encapsidated by LuIII during replication, encapsidated progeny and not cellular extracts should be used in the assays.

Literature Cited

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