REPLICATION EFFICIENCY OF THE LUIII PARVOVIRUS MINIGENOMES 3'LUIII3' AND 3'LUIII5'

By

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Abstract

Parvoviruses are currently being studied for use as possible genetic vectors in gene therapy. They are small, non-enveloped viruses of icosahedral structure, and possess a single-stranded DNA genome of approximately 5,000 base pairs that replicate only during the S-phase of the host cell. The viral replication strategy resembles the rolling circle replication model requiring the viral protein NS1. This protein functions as a site specific DNA binding protein, thus, binding the viral origin and initiating self-replication process. Studies have shown that genetic constructs with two viral terminal palindromic structures are sufficient for autonomous replication of the molecule in the presence of NS1. To compare the replication of a minigenome containing two left termini with that of one containing a left and right palindrome of LuIII the DsRed gene from the pCMV-DsRed (Clontech) was inserted between the hairpins of both vectors. The red fluorescent protein (DsRed) allowed monitoring of the replication of these constructs. The results show that LuIII 3'-DsRed-3' and LuIII 3'-DsRed-5' were both capable of expressing fluorescence in the cells over time, thus, indicating that both recombinant plasmids were capable of self replication. The results also suggest that replication of LuIII 3'-DsRed-3' was more efficient than LuIII 3'-DsRed-5'. This result suggests that a LuIII genetic vector containing two copies of the 3' hairpin (identical palindromic sequences) may be more efficient than a vector containing the LuIII 3' and 5' palindromes. This research has been helpful in providing insight about the replication efficiency expected, based on the response of the 3' and 5' terminal hairpin arrangements in genetic vectors used in gene therapy.

Resumen

Los parvovirus están siendo estudiados actualmente como posibles vectores genéticos en la terapia genética. Estos virus son pequeños, sin envoltura, con estructura icosahedral y poseen un genoma de ADN de cadena simple de aproximadamente 5,000 pares de bases que se replica sólo durante la fase S de la célula hospedera. La replicación de los parvovirus se asemeja al modelo circular de replicación el cual requiere la asistencia de la proteína NS1. Esta proteína se enlaza de manera específica al origen de replicación e inicia el proceso de replicación. Los estudios han demostrado que las construcciones genéticas con dos terminales palindrómicos virales son suficientes para la replicación autónoma de la molécula en la presencia de NS1. Para comparar la replicación de un minigenoma que contiene dos palindromes izquierdos con otro que contiene un palíndrome izquierdo y otro derecho de LuIII y el gen de DsRed de pCMV-DsRed (Clontech) se insertó entre los terminales en ambos vectores. La proteína fluorescente roja (DsRed) nos permitió el monitoreo de la replicación en estas construcciones. Los resultados mostraron que LuIII 3'-DsRed-3' y LuIII 3'-DsRed-5' son capaces de inducir fluorescencia en las células con respecto al tiempo, indicando así que ambos plásmidos recombinantes fueron capases de auto-replicación. Los resultados también sugieren que LuIII 3'-DsRed-3' tienen una mejor eficiencia de replicación que LuIII 3'-DsRed-5'. Estos resultados sugieren que de construirse un vector genético del parvovirus LuIII éste seria más eficiente si tiene dos copias del terminal 3' (secuencias palindrómicas idénticas). Esta investigación ha sido útil para proporcionar una visión sobre la

eficiencia de replicación esperada, basada a la respuesta de los terminales 3 'y 5' en vectores genéticos utilizados en la terapia génica.

I dedicate this work to my family: My parents Justino and Madeline, my husband Eric, my beautiful son Eric Alexander and finally to God, for providing me with the strength and support throughout all these years.

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CHAPTER I

Introduction

The *Parvoviridae* family consists of viruses that are small, naked, icosahedral and are currently being studied for human gene therapy (Maxwell, 2002). This family can infect cells from a range of animal species and humans. They are characterized by a number of morphological and physicochemical properties as well as distinct features of genome organization and replication (Maxwell and Maxwell, 2002). The most characteristic feature of parvoviruses is the structure of their DNA. This molecule is linear and has about 5,000 nucleotides in length. Both ends of the DNA molecule are folded back to form short double stranded, hairpin like structures (Rommelaere, 2000).

LuIII is an autonomous parvovirus that has the same genomic organization of the rodent parvoviruses. LuIII encapsidates the minus strand of the genome and has different 3' and 5' terminal sequences. It is compared with minute virus of mice (MVM) and Hamster virus (H-1). The genome organization of LuIII when compared to the rodent parvoviruses differed in two regions (Diffoot et al., 1993). An AT-rich region of 47 bp long, found as a direct repeat in MVM and H-1, is present once in LuIII and it's sequence is believed to function as an internal origin of replication. The sequences of the terminal palindromes in parvoviruses are necessary for replication. For this reason, models have proposed in which the left and right end hairpins act as origins of replication (De Jesús Maldonado, 2004) and are important in minigenomes of these viruses.

The minigenomes containing two copies of the left end palindrome of LuIII (nt 1-277) and one copy of the left end a copy of the right end palindromes, have been constructed previously. These constructs was used to construct LuIII 3'-DsRed- 3' and LuIII 3'-DsRed- 5'. A red fluorescent protein (DsRed -reporter gen) was inserted between the hairpins in both clones. The LuIII 3'-DsRed-3' and LuIII 3'-DsRed-5' plasmid will then be transfected into human cells to compare replication efficiency of both minigenomes.

The use of LuIII as a transient genetic vector for therapeutic purposes can only be accomplished by elucidating the basic biology of the parvovirus genus. This investigation aims to understand the combination of the terminal hairpins in replication.

CHAPTER II

Literature Review

Autonomous parvoviruses (APVs) are found in many species and they do not require a helper virus for replication but they do require proliferating cells and in some cases, tissue specific factors (Maxwell, Terrell and Maxwell, 2002). APVs are some of the smallest viruses found in nature and their latin name *parvus* means small. Their linear genomes terminate in little imperfect palindromes that fold back on themselves to form complex hairpin telomeres which play an important role in the modified form of rolling circle replication (RCR) (Cotmore and Tattersall, 1995). Using its terminals hairpin as a primer for DNA synthesis, the linear parvovirus genome is amplified through a series of double-stranded, monomeric, and concatemeric replicative intermediate DNA forms by a unidirectional, leading strand-specific fork. The replication takes place in the nucleus of the host cell. The virus replication is during the S and early G-2 phase of the cell cycle (Cotmore and Tattersall, 1987).

Classification

The *Parvoviridae* family is divided in two subfamilies: *Densovirinae* and *Parvoviridae*, which infect vertebrates and invertebrates respectively (Berns, 1996., Cotmore and Tattersall, 1987). The *Densovirinae* are invertebrate viruses that infect insects, they are divided into four genus: *Densovirus, Iteravirus, Brevivirus* and *Pefudensovirus.* The *Parvoviridae* subfamily includes the genera *Dependovirus,*

Erythroviru, Amdovirus, Bocavirus and *Parvovirus***.** All members are similar in clinical symptoms caused, structure, replication, but different in host range, gene expression and pathogenesis (Berns, 1996; Faisst and Rommelaere, 2000). Erythroviruses are named for their tropism for red blood cell progenitor cells. These include human parvoviruses B19 and V9, and the simian parvovirus (SPV) (Cossart et al., 1975). B19 is found to cause the common childhood disease erythema infectious and the replication is restricted to the erythroid progenitor cells in the bone marrow, it is not clear how the virus causes destruction of other cells. This parvovirus particularly spreads from person to person, just like a cold, often through respiratory secretions and hand to hand contact. Once the rash appears, the person with the illness is no longer considered contagious and does not need to be isolated.

Only members of the *Dependovirus, Bocavirus* and *Erythrovirus* genera are known to infect humans. They regulate gene expression, DNA replication and to have site specific integration, rescue from the integrated state and packaging. AAV is identified as a parvovirus that replicates exclusively in cells co-infected with a helper virus. In the absence of helper functions, AAV enters the latent pathway integrating the virus, therewith, threatening the host cell with insertional mutagenesis. AAV has acquired the unique feature of integrating in a site specific manner into a locus at human chromosome 19q13.3-qter (Lusby et al., 1980; Labow and Berns, 1987).

Bocavirus and *Amdovirus* are new genera, recently created by International Committee on Taxonomy of Viruses Virus Index Database (ICTVdB, 2005, www.danforthcenter.org_iltab_ictvnet).

The *Amdovirus* genus includes aleutian mink disease virus. The VP1 N-terminus, is shorter than those of other parvoviruses, and, lacks a phospholipase A2 enzymatic core, which is required for viral infectivity (Zadori et al., 2005).

The *Bocavirus* genus includes bovine parvovirus type 1 (BPV), canine minute virus, and the recently identified human bocavirus (HBoV) (Schwartz et al., 2002; Qiu et al., 2007). HBoV is a parvovirus that causes lower respiratory tract infections in humans and it has been linked to gastroenteritis. All members of the Parvoviridae subfamily, have two major ORFs, however *bocavirus* has a third middle ORF, which encodes a nonstructural nuclear phosphoprotein NP1 of unknown function (s) (Qiu et al., 2007).

The *Parvovirus* genus, like feline panleukopenia virus (FPLV), canine parvovirus (CPV) and aleutian disease of mink virus causes serious diseases. They are autonomous, lytic parvovirus (APVs), and only the DNA strand that is complementary to the viral messenger RNA is encapsidated. The virion consists of an icosahedral protein capsid that contains the DNA molecule, it is very resistant surviving alcohol or ether treatment, temperatures of up 56˚C and pH changes between 3 and 9. Porcine parvovirus (PPV) is another member of the parvovirus genus. PPV is the main etiologic agent of the SMEDI syndrome (stillbirth, mummification, embryonic death, and infertility), a significant cause of reproductive failure in pig. Minute virus of mice (MVM), H-1 and LuIII are classified as rodent parvovirus. LuIII was isolated as a contaminant of a human lung carcinoma (Berns, 1996).

Genome Organization

The parvovirus genus share similarities in their genome organization. The parvovirus chromosome is a linear, non-segmented, single stranded DNA strand of approximately 5,000 bases in length. This DNA can be of positive or negative polarity. The viral particles are icosahedral, 18-26 nm in diameter and the genome consists of genes that encode proteins involved in transcription (NS or REP) and another that encodes the coat proteins (VP proteins). The linear DNA plasmid has palindromic sequences, which can fold back on themselves to form two secondary structures (hairpin) that are stabilized by self-hydrogen bonding. These "hairpin" structures contain the *cis*-acting signals indispensable for genome replication. These include the viral inverted repeat (ITR) sequences that serve as the origin of DNA replication and can be different within individual genomes and species (Tam and Astell, 1993).

Previous studies have established that terminal palindromes are essential for successful replication of the human parvoviral genome. All parvovirus termini contain palindromic sequences. The human B19 virus, AAV and all Dependoviruses characterized have identical DNA sequences at both termini and the other parvoviruses characterized have different 3’ and 5’ terminal sequences.

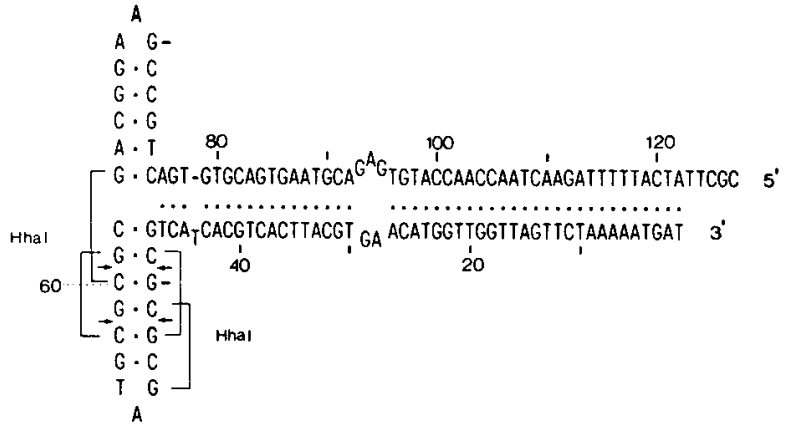
The adeno-associated viruses (AAV) and B-19 each have identical inverted terminal repeats of 147 bases long. These ITRs have the potential of forming a T-shaped secondary structure by folding the terminal 125 self complementary nucleotides (Lefebvre et al., 1984; Berns, 1996). The ITRs sequences are signals for

encapsidation, integration into the cell genome and rescue from either host cell chromosome or recombinant plasmid.

The hairpins of Autonomous Parvovirus differ in size and sequence (Berns, 1996). The 3' terminus at the left end contains 115 to 122 bases long and can assume a rabbit ear (Y or T-shape) structure. The 5’ terminus at the right end contains 206 to 242 bases long and can assume a rabbit ear (Y or T-shape) or a hairpin (U-shape) structure. When the 3’ hairpin structures of some autonomous parvoviruses are compared to a adeno-associated virus they have identical DNA sequences and consist of approximately 116 nucleotides in length (Astell et al., 1985).

The terminal palindromes exhibit orientations called *flip* and *flop* due to a few mismatched nucleotides (Astell et al., 1985). These mismatched nucleotides within the hairpin stem form a “bubble” structure that consists of a triplet 5’ –GAA in one arm of the palindrome paired to a 5’ –GA doublet in the other arm (Cotmore and Tattersall, 1987). The 3’ terminus of the minus strand of LuIII exists only in the “flip” conformation, a GAA sequence is opposed to GA sequence, and can assume a T-shape configuration (Diffoot et al., 1989) (Figure 1 A). The 5’ terminus of the minus strand of LuIII exists in both “flip” and “flop” conformations, a TCC sequence is opposed to TC sequence, and has a U-shape configuration (Diffoot et al., 1989) (Figure 1 B). Both conformations (left end and right end) have been related to the DNA replication of parvovirus. (Cotmore and Tattersal, 1988; Berns, 1996 and Chem, 1988).

A.



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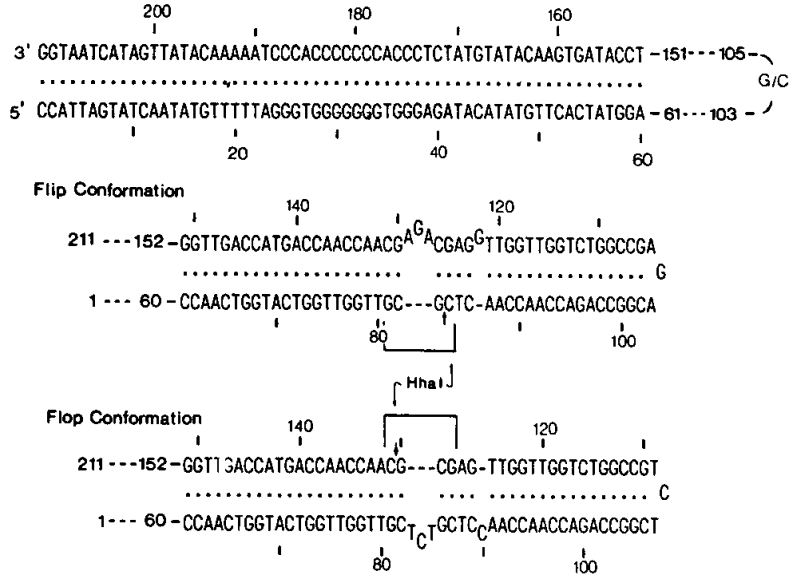


Figure 1. Palindromic Termini of Parvovirus LuIII. A. DNA sequence of the left terminus (3'end) of the minus strand of LuIII forming its characteristic T-shape secondary structure. B. DNA sequence of the right terminus (5'end) of the plus strand of LuIII forming the U-shape secondary structure with its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

Viral Transcription and Proteins

For transcription, parvoviruses use the double-stranded viral DNA in the nucleus of the infected cell as template. Transcription goes from left to right and starts at two promoters. Termination occurs close to the right end of the DNA (Berns, 1996).

The parvovirus genome has led to the evolution of a variety of strategies for the expression of all of the proteins necessary for a productive infection. The mechanisms involved include multiple promoters, splicing, alternative splice donor and acceptor sites, use of an initiation codon, and proteolytic cleavage (Berns, 1990). All proteins are encoded in one strand: the left half of the genome encodes regulatory proteins, and the right half encodes structural proteins. The autonomous parvovirus MVM and LuIII are organized into two open reading frames (ORF's) under the control of two promoters, P4 located at map unit 4, and P38 located at map unit 38 (Figure 2 and 3) (Diffoot et al., 1993). The left hand ORF, under the control of the P4 promoter, codes for the two non-structural proteins NS1 and NS2. The right hand ORF of the APVs drives the synthesis of the various capsid polypeptides VP1, VP2 and VP3, all under the control of P38 promoter (Figure 4) (Maxwell et al., 2002; Berns, et al., 1996).

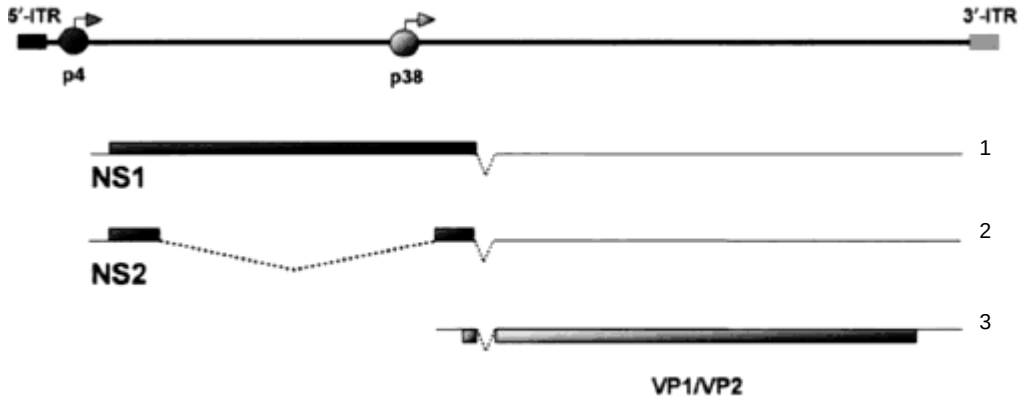


Figure 2. Minute virus of mice (MVM) transcriptional map. The genome is organized into two open reading frames (ORF). The location of the two promoters P4 and P38 is shown by arrows. The ITR's (inverted terminal repeat) are shown by boxes at both sites. 1 is showing the major transcript class that encodes for the non-structural protein NS1. In 2, two open reading frames are used to encode for the non-structural protein NS2. 3 indicates the ORF used to encode for the two structural proteins, VP1 and VP2. Alternative splicing is shown by the doted line. (Adapted from Wong and Chatterjee, 2002)

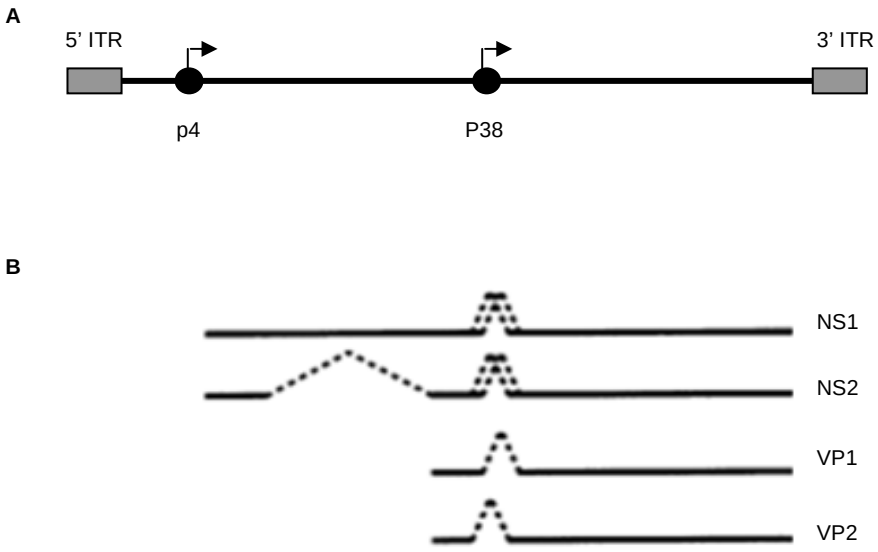


Figure 3. Structure of LuIII Parvovirus Genome. A: Genome organization. **B:** Transcripts. Transcription initiates from two promoters P4 and P38 shown by arrows and terminates at a common poly A signal. Alternative splicing is shown by doted line. The P4 and the P38 transcripts generates mRNAs encoding nonstructural proteins NS1 and NS2 as well as capsid proteins VP1 and VP2 respectively. (Adapted from Maxwell et al., 2002)

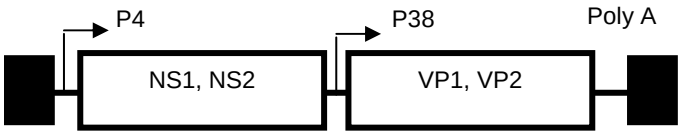


Figure 4. Structure of the LuIII and MVM Parvovirus Genome. Arrows indicate the transcription, initiates from two promoters designated P4 and P38; Black square: non identical terminal inverted repetitions. (Adapted from Maxwell et al., 2002)

B19 initiates transcripts from a promoter at map unit 6, drives both the synthesis of the major non-structural protein NS1 and the capsid proteins VP1 and VP2 (Berns, 1996). AAV has three promoters located at map units 5, 19 and 40, which drives the synthesis of four non-structural proteins, Rep proteins and capsid proteins.

In general, NS1 and NS2 are involved in replication and cytotoxicity. NS1 has a molecular weight of 83 kD and NS2 of 24 kD. The C-terminal half of NS2 is coded in a different reading frame by a region of about 35pb. This same region of the genome codes for NS1 in the original reading frame. NS1 is the most important non-structural protein, essential for a productive replication fork. This protein has a site-specific protein, recognizing the sequence (ACCA) 1-3 present in the viral origins. NS1 is dependent on protein kinase C phosphorylation. The NS2 is an auxiliary regulatory protein, which contributes to cell-type specificity and consists of three isoforms, NS2-P, -L, and –Y, that differ at their carboxy termini as a result of alternative splicing events. NS2 play a critical role in viral capsid assembly and in mRNA translation (Cotmorem et al., 1997).

NS1 also transactivates P38 which drives the synthesis of the capsid proteins, VP1 and VP-2 towards the end of the viral cycle. Parvovirus particles contain two or three different structural proteins of various molecular weights of approximately 83 and 64 kDa, but with overlapping amino acid sequences (Pujol et al., 1997; James et al., 2004; Hickman et al., 2004). VP1 and VP2 share the majority of their sequence; however VP1 has a unique N-terminal region of approximately 140 amino acids. This region includes a nuclear localization signal (NLS) (Tullis et al., 1993; Duppont et al., 1994; Faisst and Rommelaere, 2000; Lombardo et al., 2002) and a phospholipase A2 activity (Zadori et al., 2001), believed to be necessary for delivery of an infecting viral genome to the cell nucleus (Cotmore and Tattersall, 1987; Maxwell and Maxwell, 2004). VP3 is derived by proteolytic cleavage which removes the amino-terminal region of VP2 and occurs only after capsid assembly and packaging of the viral genome.

Replication of parvoviruses

The DNA replication and assembly of parvoviruses occurs in the nucleus, during S-phase and are dependent on cellular functions (Berns et al., 1996; Cotmore et al., 1994).

Parvoviral DNA replication is involving the formation of hairpin structures. The first step in the replication of viral DNA is the conversion of the single-stranded parvovirus DNA to form a complete double-stranded replicative form (RF).

Replication passes through a series of monomeric and concatemeric duplex replicative-form (RF) intermediates.

Since this process can be repeated indefinitely at each end of the genome creating, multimeric duplex intermediates containing a single continuous DNA strand. Parvovirus have adapted the rolling circle replication (RCR) mechanism to amplify themselves by a unique “rolling hairpin” process, mediated by the palindromic viral telomeres (Cotmore and Tattersall, 2003).

MVM DNA Replication

Minute virus of mice (MVM) has a negative sense DNA strand containg a 4.8 kb single strand coding region flanked by hairpin duplexes of 120 and 248 nucleotides at its 3’ (left end) and 5’ termini (right end). The mechanism of viral replication postulates that the growing strand replicates back on itself, producing a tetrameric conformation from which two plus strands and two minus strands are generated by endonuclease cleavage (Berns, 1990; Burnett et al., 2006). The single-stranded virion DNA gets converted into a closed, double-stranded, monomeric replicative form (cRF) by extension of the left terminal hairpin and ligation of the growing strand to the folded-back right terminal hairpin (Figure 5) (Cotmore et al., 1989; Baldauf et al., 1997).

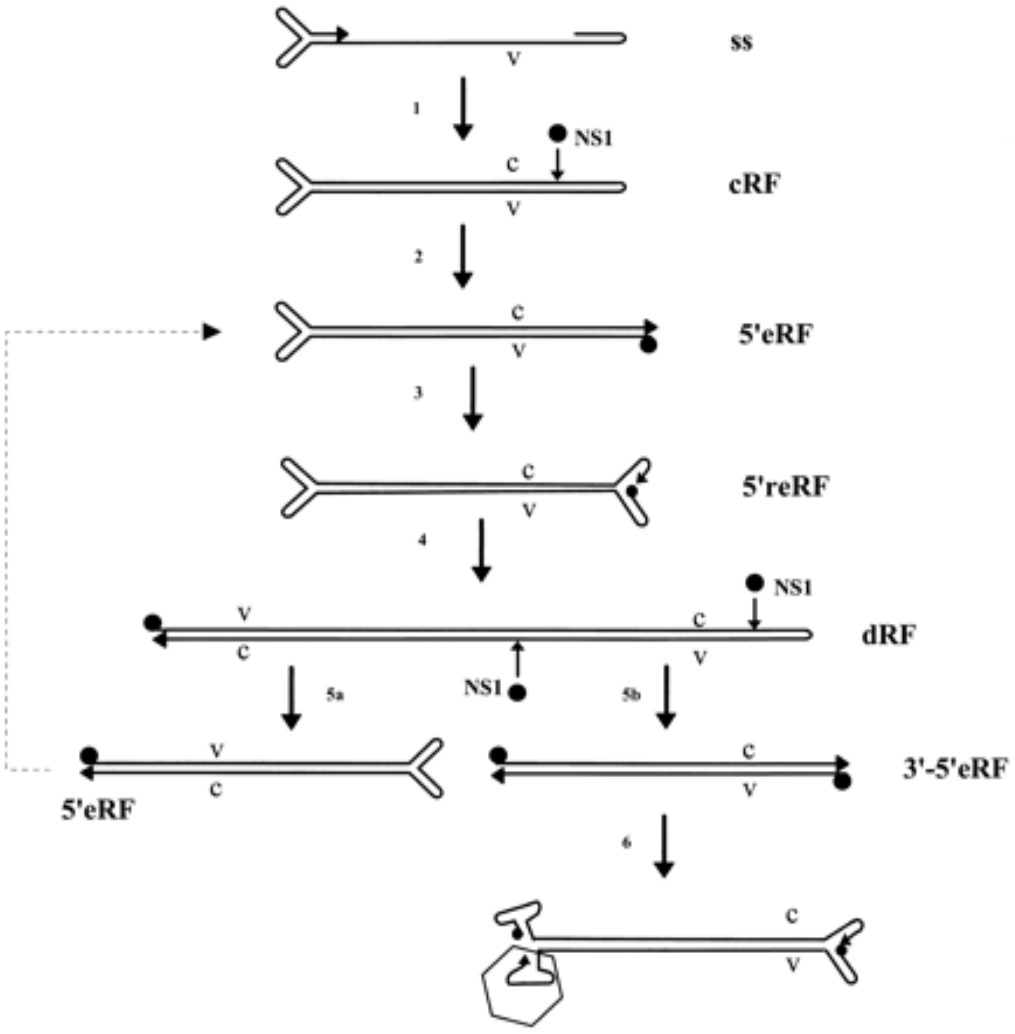


Figure 5. Modified Rolling Hairpin Model for MVM DNA Replication. NS1 is shown as a small filled circle. Small arrowheads indicate DNA 3' ends. ss, ssDNA; cRF, covalently closed RF (replicative form); eRF, extended monomeric RF; reRF, rabbit-eared RF; v, viral strand; c, complementary strand. The open polygon in step 6 represents the capsid (Adapted from Kuntz-Simon et al., 1999).

Additional processing of cRF DNA requires the activity of the viral non-structural protein NS1 (Cotmore and Tattersall, 1998; Cotmore et al., 2000). The protein NS1 is necessary in order to initiate DNA synthesis, and the Y-shaped 3' hairpin region serves as a primer for initiation of DNA synthesis by a cellular DNA polymerase. The opening of the cRF is given by mismatched sequences, which serve as cleavage sites for the NS1 nuclease. Subsequently, NS1 becomes covalently attached to the right termini of the viral genome, followed by initiation of displacement synthesis, copying of the right end sequence, and formation of a terminal extended molecule (5'eRF). Hairpin refolding at the extended terminus creates a structure called rabbit ear replicative form (5'reRF). This structure provides complement of the 5' terminal hairpin which then serves as a primer for strand displacement synthesis and dimeric replicative form (dRF)). The dimer intermediate is formed and the extended hairpin forms a palindromic double-stranded sequence termed the dimer junction. It bridges two unit lengths head-to-head duplex genomes. Duplex DNA occurs due to the nucleotides of the bubble sequence. Now, in the dimer junction, 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. Through replication, the dimer junction is associated into two structures by NS1, where the NS1 interaction with a cellular protein called parvovirus initiation factor (PIF) is able to introduce a single-stranded nick into the lower strand of the dimer junction to initiate replication (figure 6).

This factor is a binding site for the CREB/ATF family of host transcription factors and recognizes two tetranucleotide ACGT sequence, motifs within the ATF consensus and is located in the NS1 binding site $(ACCA)_2$ (Cotmore and Tattersall, 1995). The additional nucleotide in the GAA arm renders this origin inactive and restricts replication initiation to the TC arm of the dimer junction, causing the configuration asymmetric. The two types of monomeric replicative form (RF) DNA are covalently closed (5'eRF) or extended (3'-5'eRF) left-hand termini. The 5'eRF molecule generated in this way re-enters the cycle as in replicative form (5'reRF), while duplex-to-hairpin transition at the right-hand palindrome of the 3'-5'eRF molecule is thought to lead to the displacement of single stranded genomic DNA, which is then immediately packaged into the preformed empty capsid (Kuntz-Simon et al., 1999).

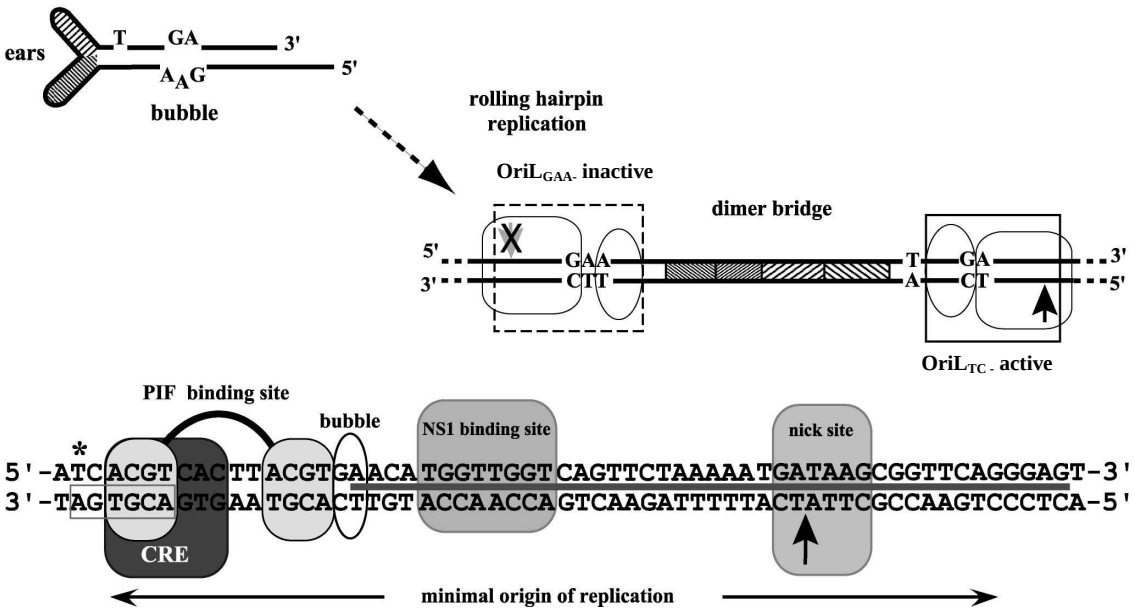


Figure 6. The dimer junction resolution and minimal origin of replication. The left-hand hairpin of MVM is shown in diagrammatic form. The small ears and bubble sequence are indicated. The dimer junction created by rolling hairpin replication, is shown on the right. Cross-hatched boxes represent palindromic sequences derived from the hairpin ears. An arrow indicates the nick site and the minimal active origin is boxed. The arrow at the potential nick site is crossed out to indicate that it is not an active origin. Sequence details of the minimal origin of replication and the bubble, PIF, CRE, and NS1 binding sites, as the NS1 nick site are shown at the bottom of the figure (Adapted from Burnett and Tattersall, 2003).

LuIII DNA Replication

The parvovirus LuIII contains cis-acting in the left hairpin sequences required for DNA replication. The left hairpin of LuIII has an active NS1 driven origin of replication that lacks the arrangement of the specific domains in the dimmer duplex intermediate proposed for MVM. It has been proposed that the plus and minus DNA strands independently initiate replication from the right and left hairpins respectively resulting in an equivalent amount of both viral DNA strands (Diffoot-Carlo et al., 2005). The possible mechanism for the replication of LuIII is shown in Figure 7, the plus strand starts replication from the right hairpin and the minus strand from the left hairpin, (step 1). This suggests that both hairpins can serve as primers, which would allow a host polymerase to synthesize a complementary copy of the internal sequence of the viral genome until the growing stand reaches the folded back terminus, resulting in a covalently closed replicative form (cRF). In step 2, NS1 opens the cRF by nicking the viral strand. These are active NSI nick sites present at both termini of parvovirus LuIII that differ from each other (Diffoot et al., 1993). These nicks generate two origins of replication running in opposite directions that lead to strand displacement. Subsequently the NS1 nick occurs and (step 3) there is a displacement and copying of the strand. In step 4 the development of an extended molecule containing covalently joint viral and complementary DNA strands occurs.

Earlier studies suggested that the left terminus of the LuIII minus strand exists only in the flip conformation, and the right terminus of the plus strand exists in both

the flip and flop conformations. This model however suggests in step 5 that in each strand both flip and flop conformations at both termini are synthesized (Diffoot et al., 2005). This difference between LuIII and MVM has implications on the replication of these viruses. The unique encapsidation pattern observed for LuIII is due to the presence of origins of replication at both the left and right termini. This explains mechanism used by LuIII for the replication of its viral genome (Diffoot et al., 2005).

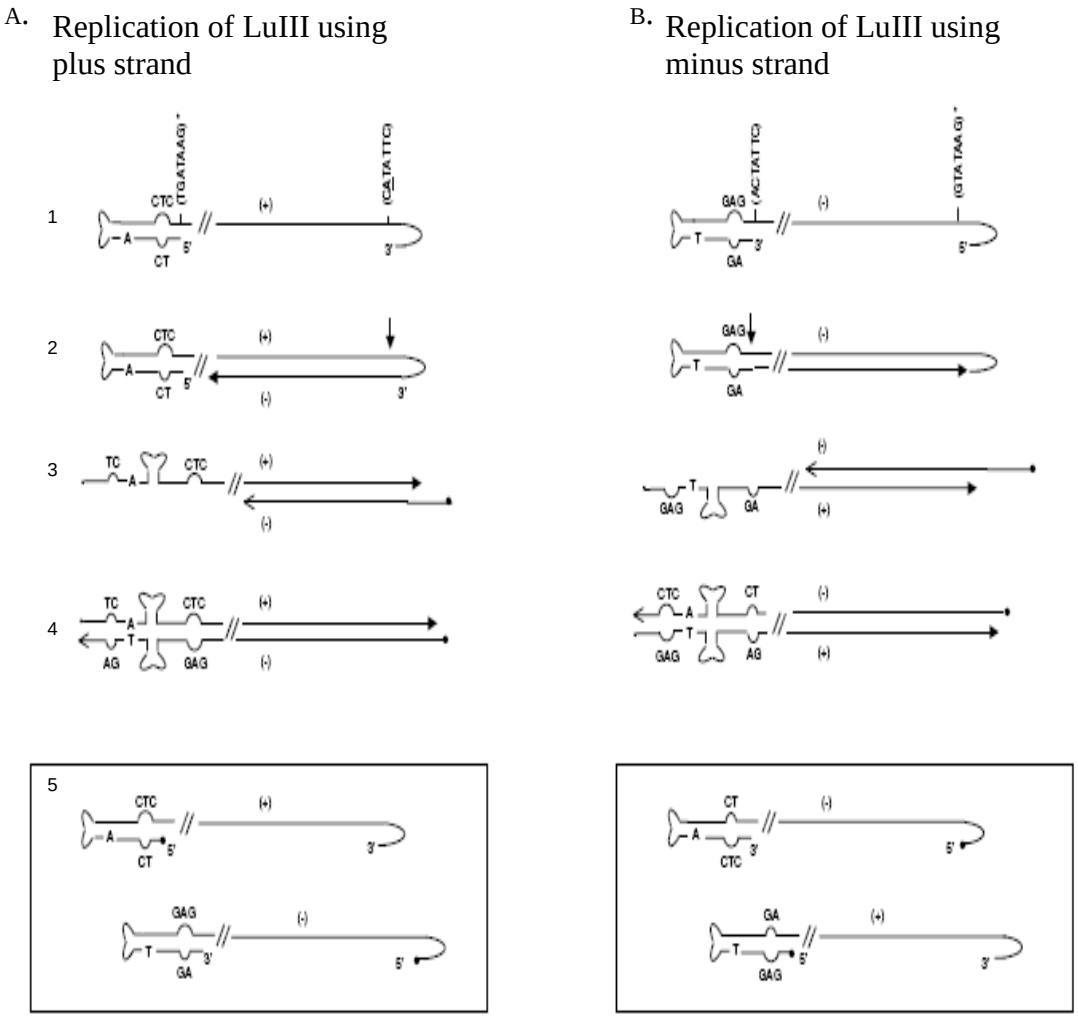


Figure 7. Proposed Model for the Replication of Parvovirus LuIII. **A:** Replication of the (+) strand, **B:** replication of the (-) strand. The NS1 nick site and its complementary sequence are indicated (*). The bubble sequence at the left hairpin is indicated. The arrows point to the NS1 nick sites. The flip and flop conformation from both strands are boxed (Adapted from Diffoot et al., 2005).

Encapsidation Pattern

All autonomous parvoviruses of known sequences have non identical ends and their encapsidation patterns allow separation into three different subgroups. The DNA encapsidated by parvoviruses can be of either plus or minus polarity and the ratio of the two strands varies among members of the Parvoviridae. Minute virus of mice (MVM), H-1 virus has related terminal sequences with over 90% homology and encapsidate 99% minus strand (Table 1). This parvovirus B-19 and LuIII have similar encapsidation patterns to that of the detective parvovirus adeno-associated virus (AAV) (Diffoot et al., 1989).

The mechanism by which LuIII encapsidates either strand of its genome remains unknown. Diffoot and colleagues (1989) proved that identical terminal ends are not required for the encapsidation of both strands since the autonomous parvovirus LuIII has non-identical termini and encapsidates both strands with equal frequency (Bates et al., 1984). Willwand and his colleagues (1993) demonstrated that empty MVM capsids bind to the left terminus of MVM replicative-form DNA, but not to the right terminus. This suggests that binding to the left end of parvovirus genomes is required for encapsidation of the minus strand and implies that binding to the right end might be required for encapsidation of the plus strand. LuIII was sequenced and has shown to have greater that 80% identity with rodent parvoviruses MVMp and H-1 (Diffoot et al., 1993). Comparison of the DNA sequences of LuIII, MVM and H-1 reveals the presence of an adenine-thymine (A/T) rich region at right end sequence that may be a determinant of the encapsidation pattern for LuIII. This region is 47

nucleotides long located at map unit 89, 6 bases downstream from the end of the right open reading frame (ORF), and is not present in H1 or MVM. It has been suggested that in LuIII, the A/T-rich region interferes with the binding of cellular proteins to the viral genome, allowing the virus to encapsidate plus and minus DNA strands with equal frequency. During 1993 and 1994, Tam and Astell identified a sequence near the right palindrome of MVM as a cis-acting replication signal. This region has shown to bind four cellular proteins which are thought to be involved with replication of the MVM genome. Corsini and colleagues (1995) determined the possible binding of cellular proteins to this region of MVM and H-1 virus, hinders binding of the MVM capsid to the right end, preventing encapsidation of the plus strand. The presenting of this protein-binding region by AT might eliminate strand-selective encapsidation and confer the facility to encapsidate either strand. If this is the situation, insertion of the 47-nucleotide AT sequence into this region of LuIII might disrupt the interactions with cellular proteins, allowing empty capsids to bind the right end and encapsidate the plus strand.

Recent studies characterized packaging complexes generated in vivo, when the right-end hairpin of MVM is replaced with that of LuIII, creating MML. The chimeric virus that encapsidates 95% of the MVM sequence is fused to the right-hand terminus of LuIII packaging 40% positive-sense DNA. MVM encounters difficulty in internalizing explicit elements in the plus strand. This is due to the virus attempts to encapsidate both positive and negative-sense genomic sequences. Full length positive sense MML DNAs were found to be rare, while partially packaged genomes

accumulated to high levels in infected cells. This indicates that the negative-strand sequence of MVM has undergone selective pressure to optimize its compatibility with the encapsidation mechanism, while the positive sequence has not (Cotmore and Tattersall, 2005). These observations suggest that for viruses that package predominantly a single strand, selective pressure is exerted on that strand to minimize the presence of unwanted elements, such as stem–loop configurations, reiterated G4 clusters, and is optimal compatibility with the translocation machinery. During viruses from the Bocavirus, Parvovirus, and Amdovirus genera (Tattersall et al., 2005; Cotmore and Tattersall, 2005), which package mostly the negative strand, stretches of G4 or longer are completely absent from the minus strand compared to the plus strand. Therefore, for example, the negative-sense single-stranded regions of MVM, AMDV, FPV, BPV, H1, and KRV are entirely devoid of such G tetrads, whereas their related positive-sense sequences contain different clusters, correspondingly. In compare, for viruses from the Erythrovirus and Dependovirus genera, which package either strand with the same efficiency, such runs of G are similarly represented on together strands (Cotmore and Tattersall, 2005). These viruses are currently receiving attention as potential experimental and therapeutic gene transfer vectors, since they allow the efficient delivery of foreign genes to a wide range of mammalian cells (Buning et al., 2004; Cornelis et al., 2004). The observations presented here provide one potential for this parvoviruses and suggest that minimizing regions of potential secondary structure in the transgene sequence may well enhance vector yield.

Scientists using genomic chimeras of the MVM and LuIII genomes established a possible connection between the mechanistic differences in the processing of $OriL_{TC}$ and OriR and the strand packaging bias of these parvoviruses (Cotmore and Tattersall, 2005). Consequently, the relative efficiency with which the two genomic termini are resolved and replicated eventually determines the polarity with which single strands are excised from the replicative form (RF), and that these are then packaged with equal efficiency. The packaging substrate is the newly released single strand and not the duplex RF forms of the genome. Further, a single-strand displacement model in which the functional asymmetries between the right and left hairpins restrict the release of positive-sense strands from the replicative form during the packaging phase of infection. For this reason, the plus strands are only released if the right end nick site is suboptimal, produced the displaced minus strands cycle through an obligatory dimer duplex intermediate (Cotmore and Tattersall, 2005).

Table 1. Encapsidation pattern of selected Parvovirus, Berns 1996.

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 % (-)

Gene Therapy

Parvoviruses are considered as suited candidates for gene therapy because they have small genomes and are capable of infecting human cells without causing clinical symptoms because of which, Autonomous Parvovirus Vectors (APV) are clearly an interesting alternative for gene therapy. Adeno-Associated Viruses (AAV) and retroviruses have been successfully studied for genetic transfer of experimental and therapeutic proteins, particularly in cancer therapy. More research is needed to fully understand these vectors, particularly with respect to mechanisms of transduction and range of tissues that can be transduced in vivo that consist of modified, capsids. The most studied vectors are viruses, among which the most promising are the small non-pathogeic viruses from the Parvoviridae family, AAV, MVM, H-1 and LuIII (Corsini et al., 1996). Some parvoviruses, including H-1 and MVM, possess onco-suppressive potential and inhibit proliferation of cells derived from malignant human tumors in culture. This tumor cells, including human neoplastic cells, can also be targets for the parvovirus onco-suppressive activity in animal's cells and destroy the surrounding tissue and eventually kill the host (Spegelaere et al., 1991).

AAV is a single-stranded DNA parvovirus with a 4.6 kb genome. Among the most viruses currently being developed as vectors for human gene therapy, AAV is one of the most promising tools because is nonpathogenic, nontoxic with low immunogenicity, and allows gene expression in various tissues. The recombinants molecules of AAVs have been successfully used in a number of animal models of neurological disorders (Burger et al., 2005). rAAV is the most commonly used

serotype and transducers both neurons and epidermal cells in the central nervous system (Davidson et al., 2000). AAV vectors can insert genetic material at a specific site on chromosome and integrated long-term expression of the transgenic suitable for human applications (Maxwell et al., 2002). There are little disadvantages to using AAV, including the small amount of DNA it can carry and the complexity in producing it. In difference to adenoviruses, many people treated with AAV will not build an immune response to eliminate the virus and the cells that have been effectively treated with it. Several trials with AAV are in the progress, mainly trying to treat muscle and eye diseases (Rabinowitz and Samulski, 2000). Therefore, are used in specific muscles using compatible promoters. Efforts are made to develop highly compact, active and yet tissue-specific promoters for use in AAV vectors (Wang B et al., 2008). AAV is considered as potential vectors for the genetic handling of lung conditions due to their natural empathy for respiratory epithelium. Other features including their ability to be prepared in gene expression and their extrachoromosomal existence has resulted in their development for the treatment of numerous other diseases. Adenovirus vectors have been revealed to efficiently infect target cell populations and to express proteins in the deficiency of toxicity (Goudy et al., 2001). In many studies, AAV, have a predilection for integration at a defined chromosomal location and may represent a safer alternative to retrovirus.

In the 1993, Diffoot and colleagues worked with LuIII vector recombinants, because of the successful infection of human cells and the highly infectious genomic clone of LuIII, pGLu883. To develop several vector systems based on the LuIII genome, the viral coding sequences of this plasmid were replaced by lacZ gene or luciferase reporter. Currently 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 (Maxwell et al., 1993). In other studies, Maxwell and colleagues replaced the P4 promotor by non-viral, regulated promoters, this resulted in maintained trans-activation responses to tissue specific or cell types (Maxwell at el., 1994). In addition, they demonstrated that LuIII recombinant genomes can be packaged in other parvoviruses capsids like those of MVM and H-1 (Maxwell et al., 2004). Replacement of the P4 promoter by bacterial tetracycline operator sequences lead to a constitutive expression in transient transfected cells and used for LuIII replication (Maxwell et al., 1999).

CHAPTER III

Objectives

The objective of this research is to compare the efficiency of the replication of LuIII 3'-DsRed-3' and of the LuIII 3'-DsRed-5' minigenomes. In order to accomplish this objective it is necessary to:

1. Construct **LuIII 3'-DsRed-3'** which will contain two identical termini (nt. 1-278) of parvovirus LuIII, flanking the reporter red fluorescent protein gene (DsRed).
2. Construct **LuIII 3'-DsRed-5'** which will contain the left (nt. 1-122) and the right termini (nt. 4924-5135) of LuIII flanking the reporter red fluorescent protein gene (DsRed).
3. Compare the replication of the minigenomes LuIII 3'-DsRed-3' and LuIII 3'-DsRed-5'.

Materials and Methods

Construction of LuIII 3'-DsRed-3'

The 3' LuIII 3' minigenome has two copies of the left end palindrome of the autonomous parvovirus LuIII (nt 1-277), cloned into the *Bam*HI site of pUC19. This clone has an insertion of a unknown segment of *Escherichia coli* sequence of approximately 300 nt in length, between the two termini (De Jesús, 2000 and Vélez, 2004). To construct LuIII 3'-DsRed-3' the 3'LuIII3' plasmid previously constructed (Vélez, 2004) sequence was digested with the restriction enzyme *PmeI* (nt 140) obtained from New England Biolabs, Beverly, MA. *Pme*I generates two fragments of ~580 and 2966 bp respectively (Figure 8). The fragment of 2966 bp corresponding to the pUC19 sequence containing both copies of the left terminus of LuIII was purified using the Gene Clean Spin Kit® (QBio-gene, No. 1101-200, Montreal, Canada). The genetic material was treated with calf intestinal alkaline phosphatase (CIAP) (Roche Applied Science, No. 10228023, Indianapolis, IN).

Independently the pCMV-DsRed plasmid (Cauliflower Mosaic Virus promoter (CMVp), (BD Biosciences Clontech, Palo Alto, CA) was digested with the restriction enzyme *Ssp*I and *Afe*I (New England Biolabs, Beverly, MA) (Figure 8). *SspI* and *Afe*I digestions generate three fragments of ~2599, 1879 and 553 bp (Figure 9B). The fragment of 1879 bp (containing pCMV-DsRed) was isolated using the Gene Clean Spin Kit®. The 1879 bp (DsRed) and 2966 pb fragments (3'LuIII3' clone lacking E. coli stuffer sequence) were then ligated with T4 DNA ligase (Roche No. 10677521,

Nutley, NJ) (Ausubel et al., 1998). The recombinant molecules were used or transformation into *E. coli* DH5α® competent cells (Stratagene, 200152, U.S.A.).

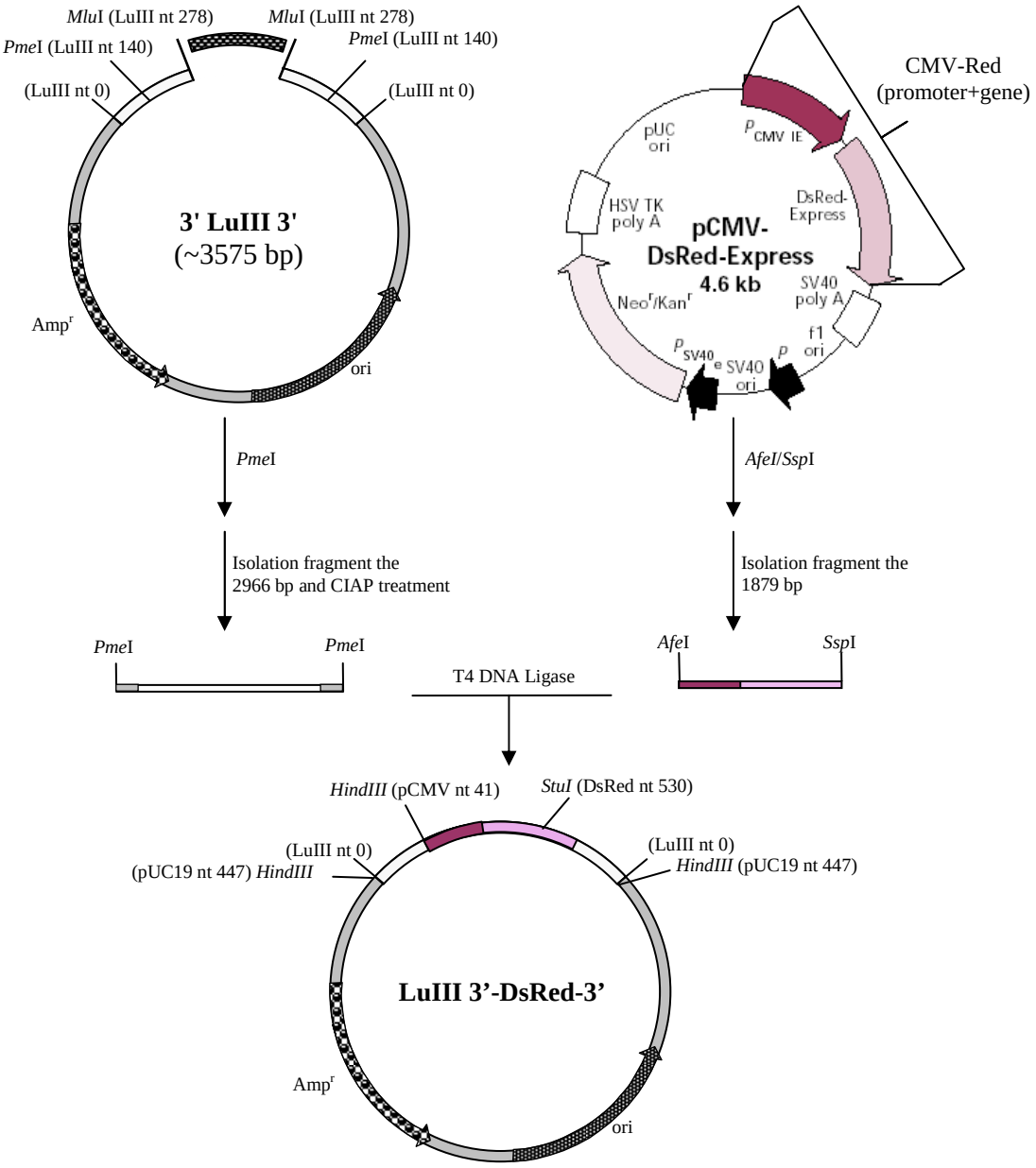


Figure 8. Strategy to construct LuIII 3'-DsRed-3'clone. Restriction sites and the nucleotide position in their respective sites are indicated.

Construction of LuIII 3'-DsRed-5'

To construct the LuIII 3'-DsRed-5'; the plasmid 3'-LuIII-5' was digested with restriction enzyme *Pme I* (GTTT/AAAC) (New England Biolabs, Beverly, MA) (Figure 9). *Pme*I linearizes the plasmid generating a fragment of 3786 bp which was extracted and purified by the Gene Clean Spin Kit® (QBio-gene, No. 1101-200, Montreal, Canada). The genetic material was treated with calf intestinal alkaline phosphatase (CIAP) (Roche Applied Science, No. 10228023, Indianapolis, IN). The pCMV-DsRed sequence was isolated independently with the restriction enzyme *SspI* and *AfeI*. The DNA fragments 3786 pb (3'LuIII5') and 1879 bp (pCMV-DsRed) were ligated with T4 DNA ligase (Roche No. 10677521, Nutley, NJ) (Ausubel et al., 1998). A sample containg all possible recombinant molecules were used for transformation into *E. coli* DH5α® competent cells.

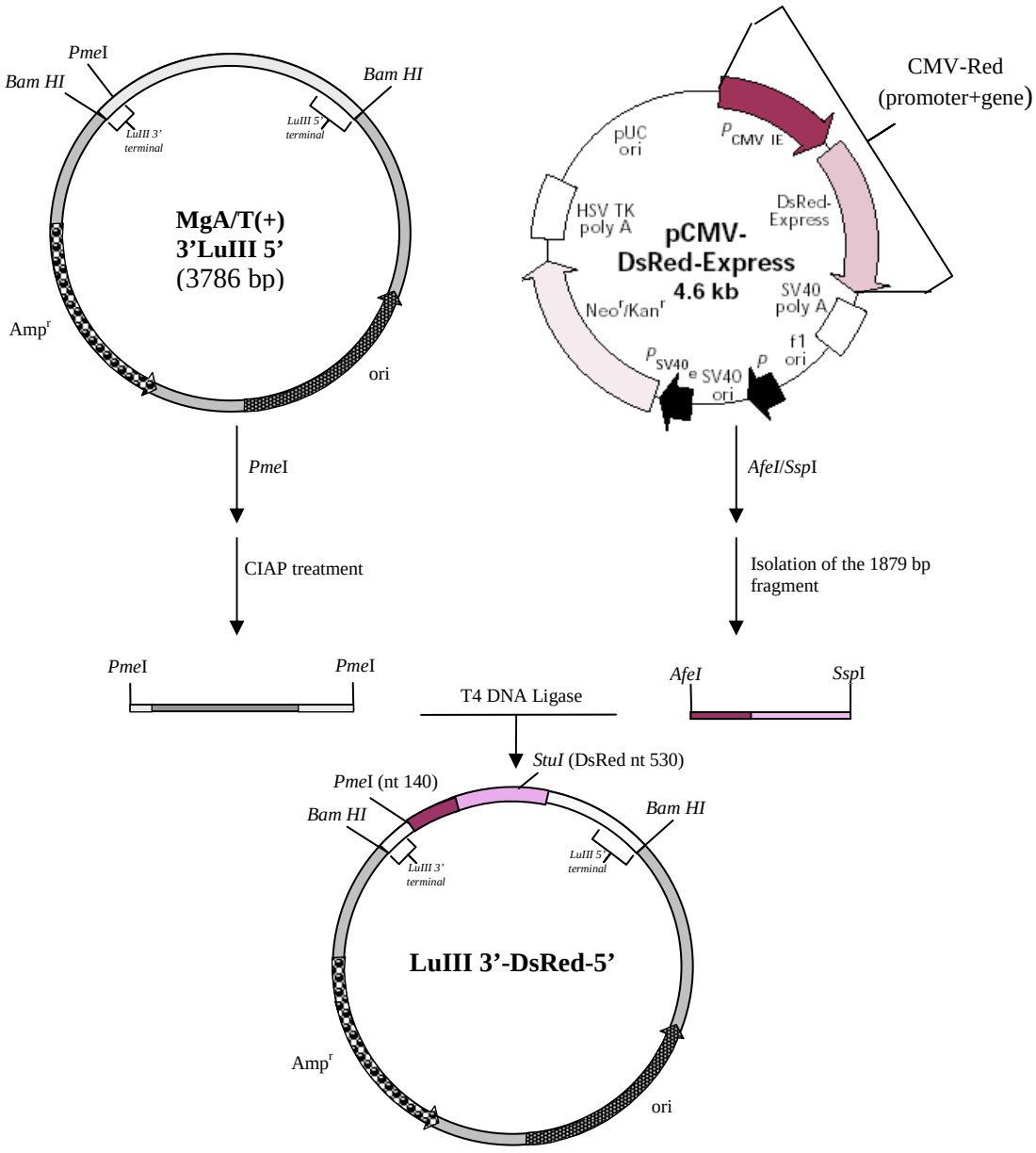


Figure 9. Strategy to construct LuIII 3'-DsRed-5'clone. Restriction sites and the nucleotide position in their respective sequences are indicated.

Preparation and Transformation of Bacterial Competent Cells by the Calcium Chloride Method

Competent cells were prepared as described by Ausubel, 2005. A single colony of *Escherichia coli* DH5α (φ80dlacZΔM15, recA1, gyrA96, thi-1, hsdR17 (rk -, mk+), supE44, relA1, deoR, Δ(lacZYA-argF)U169) strain was inoculated into 5 ml of LB (1% Bacto® tryptone, 0.5 % Bacto® yeast extract, 86 mM NaCl and 1 M NaOH) broth. The DH5α Cells were grown overnight at 37 °C with constant shaking at 250rpm. One milliliter from the culture was then transferred to 100mL LB broth and grown until an OD_{600} of 0.3-0.4 was reached. The cells were harvested by centrifugation at 1080 x g for 10 minutes at 4 °C. The pellet was resuspended in 25 ml of ice cold 50 mM $CaCl_2$ and placed on ice for 30 minutes. The cells were centrifuged at 1080 x g for 10 minutes and resuspended in 5 ml of ice cold 50 mM $CaCl_2$ in 20% glycerol. Aliquots of 100 µL were stored at - 80 °C or transferred into pre-chilled, sterile polypropylene 1.5 mL microtubes.

The recombinant molecules were transformed into DH5α competent cells. Competent cells were thawed on ice for 15 minutes. DNA was added to the cells and incubated on ice for 30 minutes. Cells were heat-shocked at 42 º C for 2 minutes bath and subsequently incubated on ice for 2 minutes. Then, 100 µL LB broth was added to the samples and incubated at 37 ºC for 1 h with shaking at 225 rpm. DH5α transformed cells were spread on LB agar plates containing the antibiotic, 50 ug/ml ampicillin. All the plates were incubated overnight at 37 °C.

The transformation of *E. coli DH5α* competent cells was done with approximately twenty-five nanograms of the respective ligation mixture. The cells with no DNA were used as a negative control, and nanograms (10ng) of pUC 19 were used for a transformation control (Table 2).

Table 2. **Transformation of *E. coli DH5*α with the products from the different ligations.**

Sample	DNA added	Amount of DNA (ng)	Plate medium
1 Control cells	None	0	LB
2 Antibiotic control	None	0	LB ampicillin
3 Standard control	pUC19	10	LB ampicillin
4-6 Phosphatase control	3'-LuIII-5'/ CIAP	20 30 50	LB ampicillin
7-9 Phosphatase control	3'-LuIII-3'/ CIAP	20 30 50	LB ampicillin
10-12 Experimental	LuIII 3'-DsRed-5'	20 30 50	LB ampicillin
13-15 Experimental	LuIII 3'-DsRed-3'	20 30 50	LB ampicillin

Isolation and Characterization of LuIII 3'-DsRed-3' and LuIII 3'-DsRed-5' Recombinant Plasmids

The resultant transformants were inoculated in 5 ml of LB broth with ampicillin, and incubated overnight at 37 °C, with constant shaking. Extraction and purification of plasmid DNA from the cultures were done using the Small-Scale Plasmid Purification Protocol (Minilysates) (Ausubel et al., 1998). The resulting clones were characterized by restriction enzyme analysis. Once the restriction characterizations were done, the molecule of each plasmid was selected to be used in the transfection assay.

Tissue Culture

The HeLa (ATCC, Rockville, MD) cells were grown in Minimal Essential Medium (MEM Eagle) (MP Biomedicals, Aurora, OH) supplemented with 10% fetal bovine serum (FBS), (HyClone, Logan, UT) and PSG (8mM Penicillin G, 3mM Streptomycin Sulfate, 200mM L-Glutamine). The cells were then incubated at 37 ºC in 25 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 2490 x g 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

The new clones were transfected by the electroporation method into HeLa cells (Maxwell and Maxwell, 1988). The HeLa cells were maintained in a 25 cm^2 plastic culture flasks in medium consisting of 90% MEM 1X with 10% FBS supplemented with PSG. Cells were incubated and harvested at a temperature of 37ºC until the desired confluence (~90%) was obtained. Then they were washed three times with PBS 1X to eliminate the serum and tripsinized at 37 ºC for 5 min. Cells were harvested by centrifugation at 5000 x g for 5 min at 4 ºC. The pellet was resuspended in 10 ml PBS-10% and centrifuged as described above. The pellet was resuspended in 7.2 ml 1X MEM and a concentration of approximately 10^7 cells/ ml (800 µl) were transferred into 1.5 ml tubes; 15 µg of DNA was added to the corresponding tubes and incubated at 37ºC for 10 min. The cells were transferred to sterile cuvettes with a 4-mm electrode separation (Eppendorf, Westbury, NY), and electroporated individually at 230 V and 960 µF using a capacitance discharge machine Gene Pulse II Electroporation System (Bio-Rad Laboratories, Hercules, California). After the pulse, 700 µl of MEM/10% FBS was added to the cuvette and the cells were mixed carefully and were immediately transferred to 25 cm^2 flasks containing 3ml warm culture medium. They were placed in the incubator at 37ºC. The flasks were maintained in the dark at all times. Medium was replaced 24 hours after transfection, the cells were observed at 24, 48, 72 and 96 hours.

Fluorescent Microscopy

Samples were observed at 24, 48 72 and 96 hours post-transfection using a Confocal Laser Scanning Microscope (Fluo View™ 300 Confocal Microscope) (Olympus, USA).

The plasmid encoding the DsRed sequence, expresseing the red fluorescent protein, could be detected using the laser S43 Green Hene and the 60SBP filter.

Flow Cytometer

The cells were washed with PBS 1X and then tripsinized at 37ºC for 5 min. Cells were harvested by centrifugation at 2110 x g for 5 min. The pellet was resuspended in 10 ml PBS 1X and centrifuged as described above. The pellet was resuspended in 1 ml PBS 1X, and counted 96 hours post-transfection using a BD FACSAria Flow Cytometer (BD Biosciences).

CHAPTER VI

Results and Discussion

Most autonomous parvoviruses contain non-identical hairpin structures at their 3' and 5' termini and encapsidate DNA strands of minus polarity. LuIII has non-identical termini, and like AAV and B19 encapsidates the same amounts of plus and minus strands. The palindromes at these termini exhibit two alternative sequence orientations recognized as *flip* and *flop*. LuIII syntheses equal amounts of *flip* and *flop* conformations at the right end of the plus and minus strands, while the left terminus of the minus strand shows exclusivity the *flip* conformation. (Diffoot et al., 1989). The aim of this work was to compare the efficiency of replication of the minigenomes LuIII 3'-DsRed-3' and LuIII 3'-DsRed-5', which have the reporter gene, pCMV-DsRed, inserted between their respective termini. This red fluorescent protein (DsRed) gene was isolated from the coral Discosoma. This represents a new marker that has been codon-optimized for high expression in mammalian cells. The replication of both constructions were analyzed qualitatively using a fluorescent confocal microscope and quantitatively utilizing flow cytometry to determine which hairpin combination, 3'-DsRed-3' versus 3'-DsRed-5' replicates more efficiently. Our results suggest that 3'-DsRed-3' replicates more efficiently when compared to the replication of 3'-DsRed-5'.

Restriction analysis to construct recombinant molecules

To construct LuIII 3'-DsRed-3', the 3'LuIII3' plasmid previously constructed (Vélez, 2004) which has two recognition sites in the terminal sequence, was digested with *Pme*I (nt 140) generating two fragments of ~580 and 2966 bp (figure 10. A). The 2966 bp fragment corresponds to the pUC19 sequence and both copies of the left terminus of LuIII. The other fragment present is the *E.coli* stuffer sequence. The 2966 bp fragment was purified and treated with (calf intestinal alkaline phosphatase) CIAP, removing the phosphate group at the 5' end of the nicked site. This treatment inhibits the formation of a phosphodiester linkage between the 3' and 5' ends of the linear vector, favoring the cloning of the DsRed insert, of 1879 bp.

The pCMV-DsRed plasmid was digested with *SspI* and *AfeI* generating three fragments of ~2599, 1879 and 553 bp (Figure 10. B). The 1879 bp (DsRed) and the 2966 bp fragments (3'LuIII3' clone lacking *E. coli* stuffer sequence) were then ligated with T4 DNA ligase (Roche No. 10677521, Nutley, NJ) (Ausubel et al., 1998). The recombinant molecules were then transformed into *E. coli* DH5α® competent cells (Stratagene, 200152, U.S.A.).

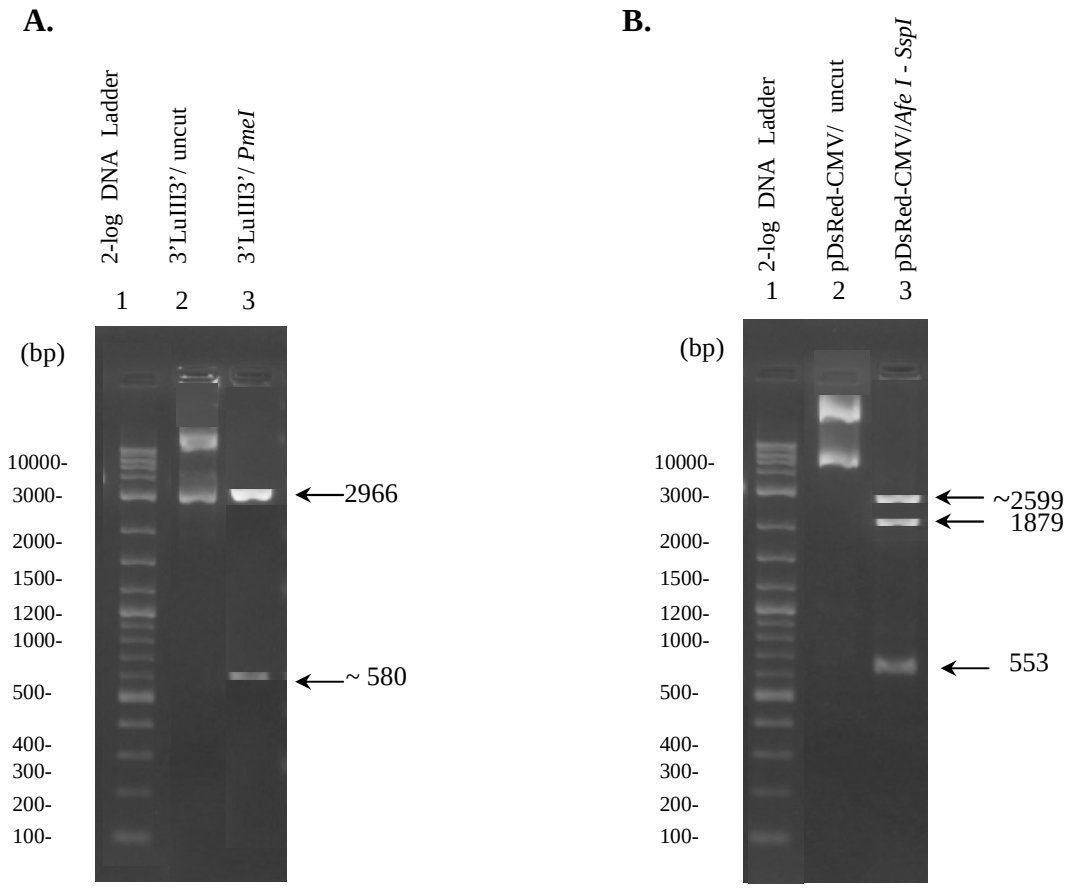


Figure 10. A. Digestion of 3' LuIII 3' with *Pme*I. New England Biolabs, Beverly, 2-log Ladder (bp: base pair). **B. Digestion of pCMV-DsRed with *AfeI/ SspI***. 1.2% agarose gel in 1X TAE buffer electrophoresed at 70 V.

To construct LuIII 3'-DsRed-5' the 3'LuIII5' plasmid previously constructed (Sanchéz, 2004) sequence was digested with *Pme*I (Figure 11). *Pme*I linearizing the plasmid generating a fragment of 3786 bp. The genetic material was treated with calf intestinal alkaline phosphatase (CIAP) (Roche Applied Science, No. 10228023, Indianapolis, IN), preventing the recircularization and religation of the linearized vector, favoring the cloning of the DsRed insert, of 1879 bp. The CMV-Red sequence was isolated and treated as described above (Figure 10.B). The DNA fragments were ligated with T4 DNA ligase (Roche No. 10677521, Nutley, NJ) (Ausubel et al., 1998). The ligation mix containing the recombinant molecules was transformed into *E. coli* DH5α® competent cells.

All DNA samples were analyzed by gel electrophoresis on a 1.2% agarose gel (Fisher, Molecular Biology Grade, BP1356100) in 1X TAE buffer (40mM Tris-acetate, 2 mM EDTA, pH 8.5) at 70 V.

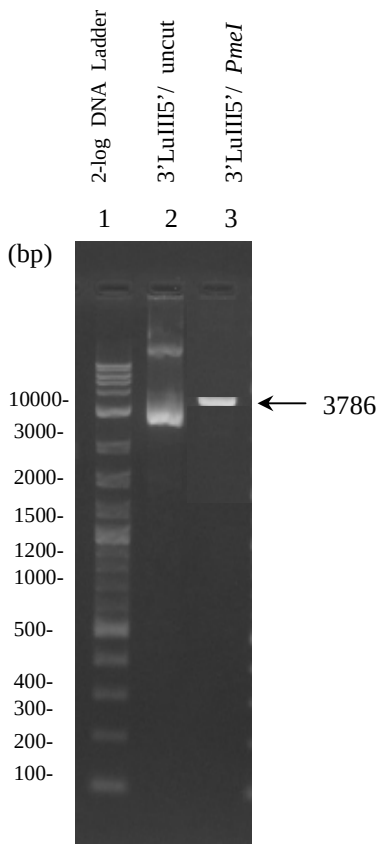


Figure 11. Digestion of 3' LuIII 5' with *Pme*I. New England Biolabs, Beverly, 2-log Ladder. 1.2% agarose gel in 1X TAE buffer electrophoresed at 70 V.

Transformation of the recombinant molecules

Transformation into the bacterial *E. coli* DH5α competent cells, were described under the Materials and Methods section. These transformations were done in independent assays with the corresponding DNA molecules (Appendix 1). In the study, fifteen transformations were performed. The control plates used in the all transformation assays resulted as follows. Normal growth of non-transformed cells was observed on the plates without the antibiotic ampicillin, indicating that the cells were viable after the heat shock. No growth of non-transformed cells was observed on the plates containing ampicillin indicating that the cells were susceptible to the antibiotic and that the cells were not contaminated. Blue colonies grew on the transformation control plates, while the white colonies grew on a selective media of the experimental samples. Since LuIII was cloned in the *BamHI* site of the multiple cloning site (MCS) of the pUC 19 vector and the MCS is inserted after the fourth codon of the *lacZα* gene, LuIII interrupts the expression of the gene. All transformations were handled similarly except with slight changes of temperature and time of the ligation reaction. T4 DNA ligase catalyzes the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA or RNA. This enzyme will join blunt end and cohesive end termini as well as repair single stranded nicks in duplex DNA, RNA or DNA/RNA hybrids (Roche No. 10677521, Nutley, NJ). T4 DNA ligase was used to join the abrupt terminal ends (blunt ends) to generate LuIII 3'-DsRed-3 'and 3'-DsRed-5'. According

to the company Roche Applied Science, T4 DNA Ligase should be used for Blunt-end ligations. This is generally efficient at temperatures between 15–20°C for 4–18 hours, while sticky ends are ligated effectively at room temperature (22°C) for 3 hours or if necessary 4–8°C overnight. These changes in the transformations were made as the colonies obtained in the first two transformations were not positive for the desired clones. Transformation number fifteen resulted in colonies of the desired recombinant plasmids; this may have been the result of a 25°C temperature and increase in time of ligation (overnight) favoring the joining of these terminal conformations.

All plasmids isolated from transfection fifteen were subjected to restriction endonuclease analysis as described in the Materials and Method section. The LuIII 3'-DsRed-3' and LuIII 3'-DsRed-5' recombinant molecules resulted from a transformation with an efficiency of 3.3×10^6 CFU/µg. All transformation assays had a low efficiency in comparison to the 10^7 CFU/ µg (Ausubel et al., 1998) expected for the calcium chloride method; this is probably due to the complexity of flip and flop conformations (left end and right end) of the palindromes present in the recombinant molecules or the attraction of a cellular protein homology such as the parvovirus initiation factor (PIF), identified in eukaryotic cells, which binds to the origin of the DNA molecules. Replication at the 3' end of the genome has been associated to the DNA replication of parvovirus (Christensen et al., 1997; Christensen et al., 2002).

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

Preparations of 3'-DsRed-5' recombinant DNA constructs were first characterized by digestion with *Bam HI*. Constructs with expected DNA fragments generated three bands of ~2686 bp, 1961bp and 1025 bp. *StuI* enzyme was also used to characterize the clones and orientation of the DS Red gene in the construct. Digestion with of *BamHI/StuI* generated four bands of 2686 bp, 1472 bp, 1025 bp, and 449 bp, fragments expected for the DsRed insert and the 3'LuIII5' vector sequence (Figure 12; lane 7-9; Table 3).

A.

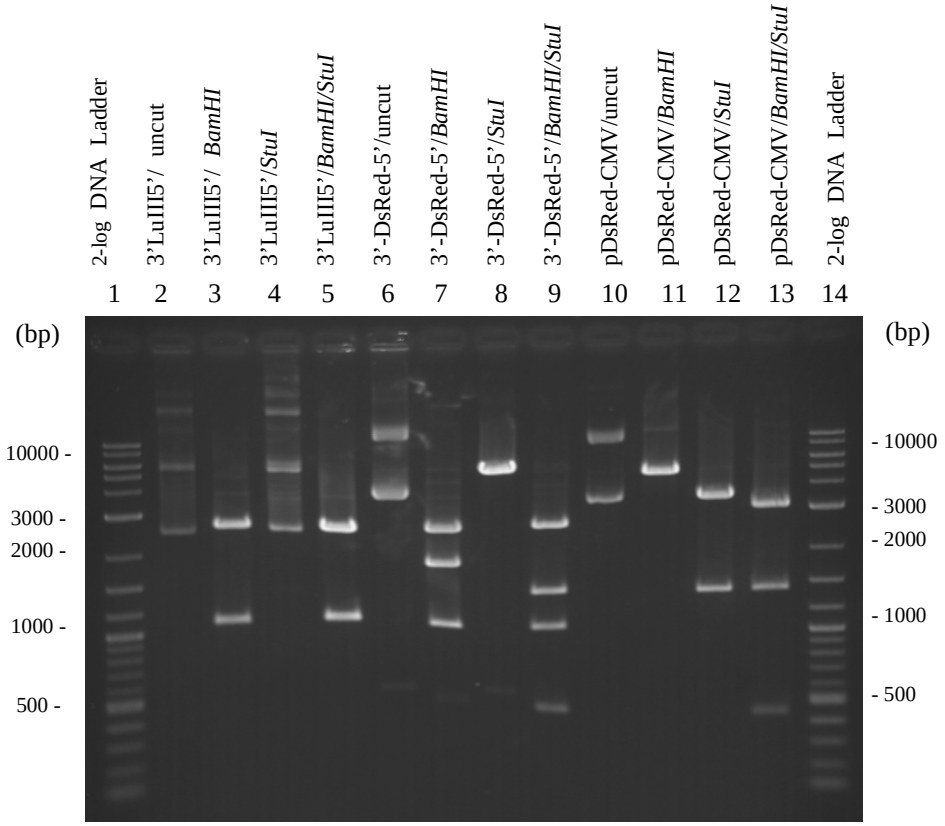


Figure 12: *BamHI* and *StuI* digestions of the recombinats 3'-DsRed-5'. A. Lanes 1 and 14: Sizes of the 2-*log* DNA Ladder indicated. Samples were electrophoresed on 1.2% agarose gel in TAE 1 X buffer at 70V.

Table 3. Expected length of the fragments generated for each plasmid when digested with selected restriction enzymes. Characterization of the 3'-DsRed-5'.

Plasmid	Restriction enzyme	Expected length fragments (bp)
3'LuIII5'	*BamHI*	2686, 1100
	StuI	Uncut
	Bam HI/StuI	2886, 1100
3'-DsRed-5' clone	*BamHI*	2686, 1961, 1025
	StuI	5672 (linearized molecule)
	Bam HI/StuI	2686, 1472, 1025, 449
pCMV-DsRed	*BamHI*	4107 (linearized molecule)
	StuI	2684,1423
	Bam HI/StuI	2684, 1423

The 3'-DsRed-3' constructs were characterized by digestion with *StuI and HindIII* (Figure 13). If the construct is well oriented and contains two copies of the LuIII-left hairpin, digestion with *StuI/HindIII* should produce four fragments of 2686, 926, 709 and 498 bp. The first fragment corresponding to the pUC19 sequence, the second fragment is pCMV-DsRed insert and third and four are fragments Left-LuIII-Left hairpins. Digestion with *StuI* should linearize the plasmid generating one fragment of 4812 bp (Table 4). Origin of replication is located at nucleotide 41 as shown Figure 8.

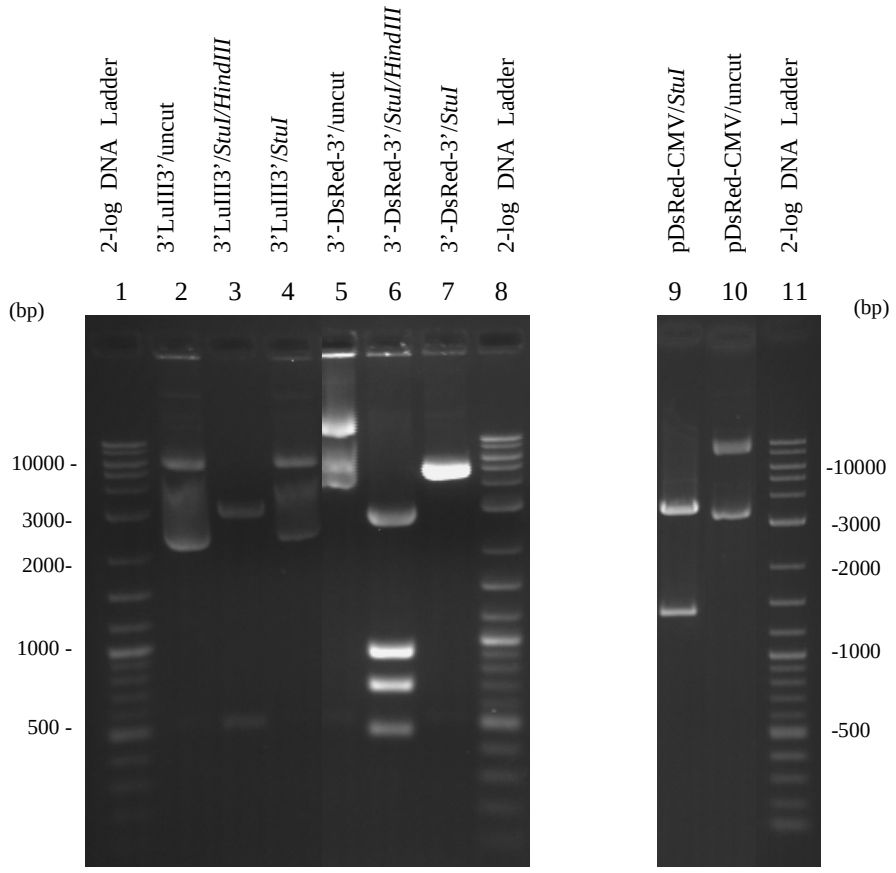


Figure 13: Digestion of 3'-DsRed-3' with *StuI* and *HindIII*. Lane 1: Sizes of the 2-*log* DNA Ladder are indicated. Samples were electrophoresed on two different agarose gel (1%) in TAE 1 X buffer at 70V.

Table 4. Expected length of the fragments generated for each plasmid when digested with selected restriction enzymes. Characterization of the 3'-DsRed-3'.

Plasmid	Restriction enzyme	Expected length fragments (bp)
3'LuIII3'	*StuI/HindIII*	2686,498
	StuI	Uncut
3'-DsRed-3' Clone	*StuI/HindIII*	2686, 926, 706, 498
	StuI	4812 (linearized molecule)
pCMV-DsRed	*StuI*	2684,1423

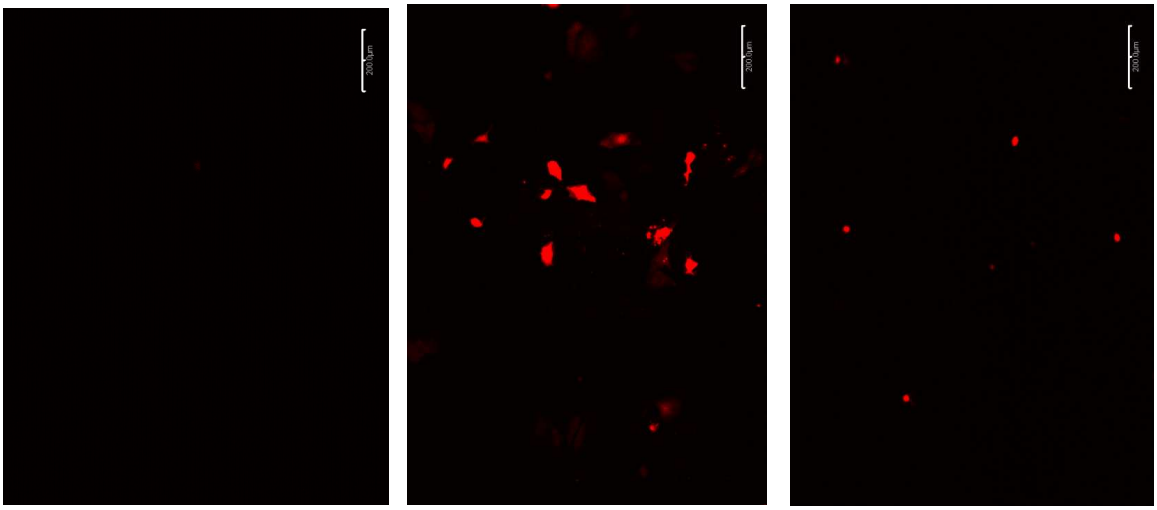
Transfection of HeLa cells and Observation in Confocal Microscope

Clones 3'-DsRed-3' and 3'-DsRed-5' were independently transfected by electroporation into HeLa cells. The total amount of DNA used is important for obtaining high transfection efficiency and is recommended DNA concentration is 2.5 to 20 µg of DNA per transfection (Bio Rad Gene Pulser® Electroprotocol). Replication of the plasmids was detected by tracking the expression of DsRed, the red-emitting cells are detected by fluorescent confocal microscopy (Excitation/Emission Maxima: 558 nm / 583 nm) (Baird et al., 2000) (Long et al., 2005). The reporter gene pCMV-DsRed is controlled by the CMV promoter for the expression of the red protein (BD Biosciences Clontech, Palo Alto, CA). The protein is found in the cytoplasm or nucleus and it is expressed independently of the replication origin. Fluorescence allows us to follow the plasmid and determine indirectly if the plasmid could replicate by itself in the HeLa cells. When the mother cells divide, the plasmid is passed on to

the daughter cells, expressing the red fluorescent protein and therefore an increase in the number of fluorescent cells is expected. If the plasmid does not have the capacity to replicate by itself, the plasmid will not be passed on to the daughter cells. Expression of the fluorescent protein will only be observed in the mother cells and not in the daughter cells, resulting in a loss of fluorescence over time. The fluorescent confocal microscope facilitated the observation of cells and the qualitative comparison of the expression of red cells with 3'-DsRed-3' and 3'-DsRed-5'.

When 3'-DsRed-3' and 3'-DsRed-5' were transfected, non-structural protein NS1 was also transfected. This protein is essential for a productive infection and replication of the virus (Legendre and Rommelaere, 1992; Cotmore and Tattersall, 1995; Cotmore and Tattersall, 1994). It is an abundant and long-lived nuclear phosphoprotein of 83 kDa with 3' -to- 5' helicase (Christensen and Tatersall, 2002) and ATPase activities (Wilson et., al 1991). 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 (Cotmore et., al 1995; Willwand et., al 1998). NS1 has been shown to also cuts minigenomes by producing the nicks described releasing the palindromic terminal sequences from genetic constructs (Vélez, 2004). Once transfected clones were observed on the fourth day of transfection (96 hours). The control cells used in the transfection assay resulted as follows: the pCMV-DsRed positive control expressed the red fluorescent protein suggesting a successful transfection and expression of DsRed. LuIII 3'-DsRed-3' and LuIII 3'-DsRed-5' without NS1 protein,

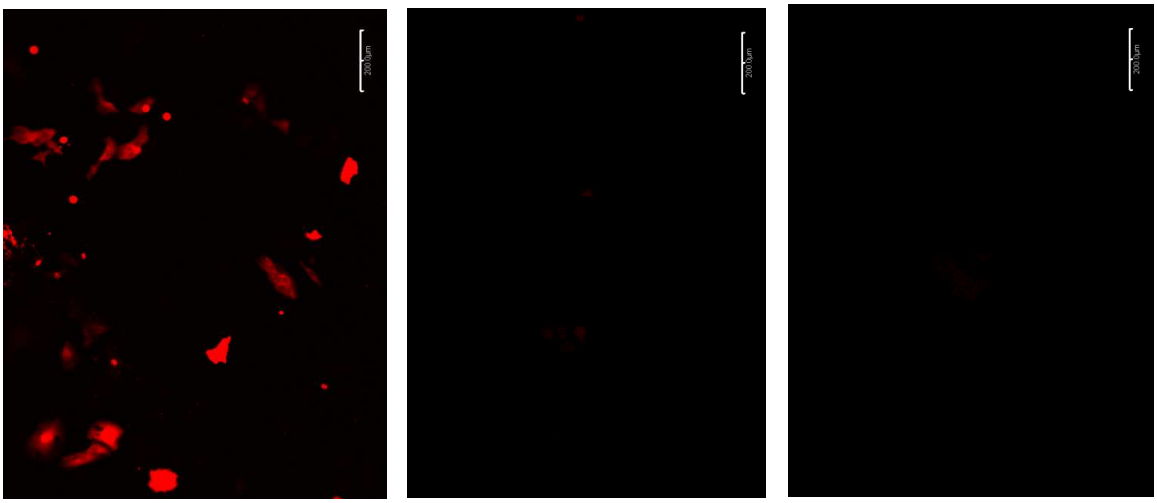
not configure the replication. The HeLa cell control plate, non transfected cells, does not have significant changes in the monolayer as expected. In the first experimental sample, cells transfected with LuIII 3'-DsRed-3' showed a expression of red cells similar to the positive control sample (pCMV-DsRed) and the second experimental sample LuIII 3'-DsRed-5' is low in the number of red cells in comparison (Figure 14) to the positive control sample (pCMV-DsRed).



Control

LuIII 3'-DsRed-3'/NS1

LuIII 3'-DsRed-5'/NS1



Positive control
pCMV-DsRed

LuIII 3'-DsRed-3'

LuIII 3'-DsRed-5'

Figure 14. HeLa Cells were photographed at 96 hours post-transfection and observed in a fluorescent confocal microscope. The cells were observed with a magnification of 10X, laser S43 Green Hene , filter 605BP and a scale bar of 200.0 µm.

Quantitative Analysis by Flow Cytometry

The transfected cells were counted at 96 hours post-transfection with a FACSAria Flow Cytometer (BD Biosciences). This equipment allows the counting and examination, of cells and chromosomes, in suspended them in a stream of fluid and passed by an electronic detection laser. Each suspended particle from 0.2 to 150 micrometers passing through the beam scatters the ray, and fluorescent chemicals found in the particle or attached to the particle may be excited into emitting light at a wavelength longer than the light source. Various detectors are aimed at the point where the stream passes through the light beam: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter or SSC) and one or more fluorescent detectors. The cell mixture was separated on the basis of the presence or absence of fluorescence. In all the experiments, a total of ten thousand cells were counted.

The normal distribution of cells is determined by their morphology, size and complexity, (Figure 15A). The cells are separated according to the presence or absence of the fluorescent protein DsRed present in the HeLa cells, (Figure 15B). In the experiments described below the population of red fluorescent cells occupy the right quadrant and the non-fluorescent cells are observed in the left quadrant. In these assays quadrants, Q1 and Q2 were not used because the cells only had red fluorescence and there was no combination of others fluorochromes. In the case that other combinations of more than one fluorochrome were used, the distribution of the cells would be, in Q1: presence of fluorochomer “Y” and absence of “G”, in Q2:

presence of “Y” and “G”, in Q3: absence of “Y” and “G”, and Q4, absence of “Y” and presence of “G”.

To compare the replication between LuIII 3’-DsRed-3’ and LuIII 3’-DsRed-5’ clones were counted by the technique flow cytometer, in the BD FACSAria Flow Cytometer (BD Biosciences) at 96 hours. The results illustrate that morphologically the cells were in good health, according to complexity and size (Figure 16). The results were expressed as percentage (%), as a ratio of fluorescent cells with respect to the total cell count (Table 5). This experiment was repeated three times.

In the experimental sample LuIII 3'-DsRed-3’ had an expression of 473 red cells similar to that of the pCMV-DsRed positive control sample (523). The experimental LuIII 3'-DsRed-5’ had a lower expression of ds-red cells (221) in comparison to the positive control (523). LuIII 3’-DsRed-3’ and LuIII 3’-DsRed-5’ without NS1 protein, not express the red cells. These results suggest similar findings when compared to the observations from the confocal fluorescence microscope.

A. B.

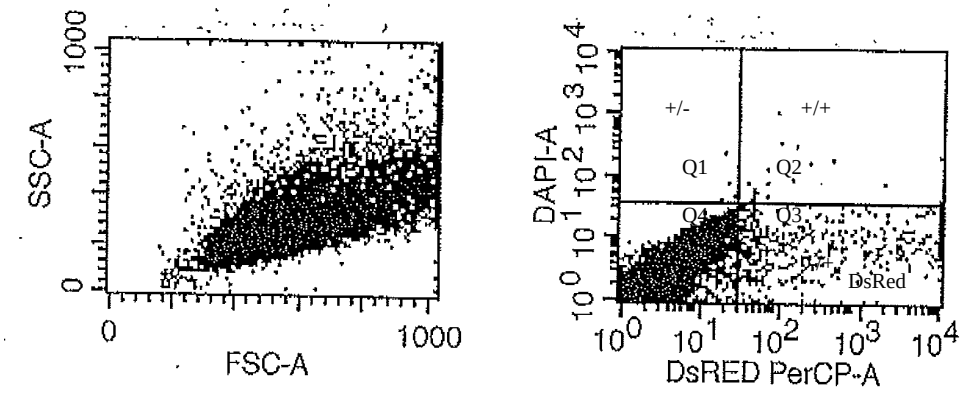


Figure 15: Example of spatial distribution of cells generated by the flow cytometer. A. Quadrant four shows the distribution of cells according to their size and viability, with a total of ten thousand cells counted. **B**. Distribution of Hela cells according to the presence or absence of the fluorescence (DAPI-A: effect DsRed protein that show a DNA specific probe for flow cytometry; PerCP-A: peridinin chlorophyll protein).

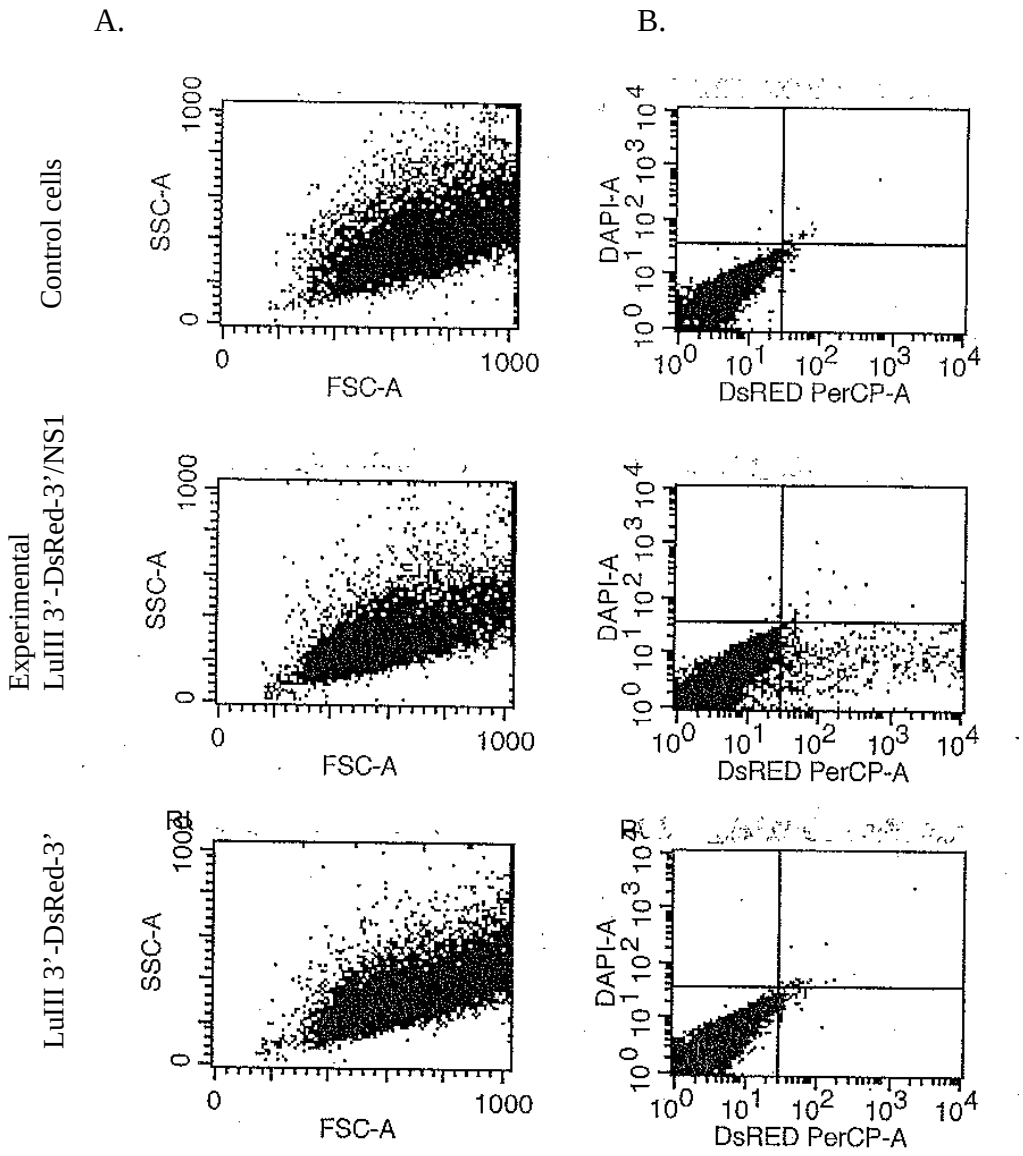


Figure 16: Spatial distribution of HeLa cell population after 96 hours post-transfection in the flow cytometer instrument. This includes: 1-control cells: HeLa cells, non tranfected cells, 2-experimental: LuIII 3'-DsRed-3'/NS1, 3-LuIII 3'-DsRed-3' without NS1 **A**. Distribution of cells according to their size and viability.**B**. Distribution of cells according to the presence or absence of DsRed protein.

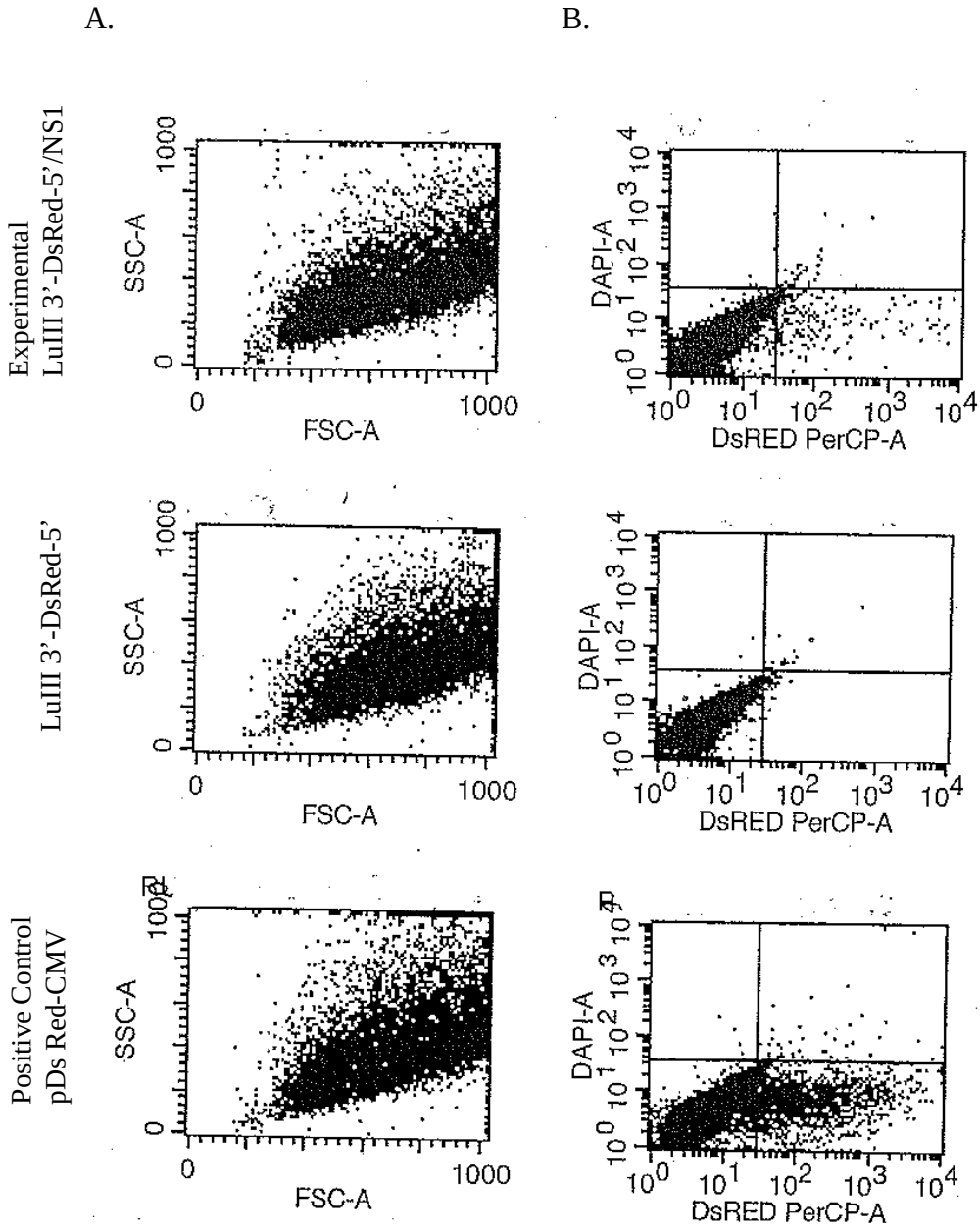


Figure 16: Spatial distribution of HeLa cell population after 96 hours post-transfection in the flow cytometer instrument. 4-experimental: LuIII 3'-DsRed-5'/NS1, 5-3'-DsRed-5' without NS1 and 6-positive control: pCMV-DsRed fluorescent protein. **A**. Distribution of cells according to their size and viability. **B**. Distribution of cells according to the presence or absence of DsRed protein.

Table 5. Percentage of fluorescent HeLa cells after transfection with plasmids indicated.

Plasmid	Cells	# cells	%	Average of fluorescent HeLa cells	Standard deviation of Average
Cell control	NF	9929	99.29	43	17
	F	51	0.51		
Experimental LuIII 3'-DsRed-3'/NS1	NF	9501	95.01	439	30
	F	473	4.73		
LuIII 3'-DsRed-3'/ without NS1	NF	9864	98.64	109	5
	F	103	1.03		
Experimental LuIII 3'-DsRed-5'/NS1	NF	9726	97.26	200	22
	F	221	2.21		
LuIII 3'-DsRed-5'/ without NS1	NF	9902	99.02	65	7
	F	73	0.73		
pCMV-DsRed Positive control	NF	9430	94.30	536	26
	F	623	6.23		

*NF: Non Fluorescent, F: Fluorescent

The experimental LuIII3'-DsRed-3'/NS1 cells had higher florescence in comparison with the LuIII3'-DsRed-5'/NS1. That is possible due to the combination of the terminal hairpins, the presence of non-identical hairpin structures and may confer different stability. The positive control pDsRed-CMV had a constant low number of cells; this is possible due to the fact that cells were observation at 96 hours post transfection. According to the company Clontech the pDsRed protein is detected twelve hours after transfection. Our studies were observed at 96 hours post-

transfection. During earlier attempts these experiments were observed at 12, 24, 48, 72 and 96 hours, but DsRed was slightly observed at 12 hours for all samples. The intensity increased over time (12, 24, 48, 72, 96 hours) with the highest intensity observed at 96 hours post-transfection. For this reason the experiments described here were performed at 96 hours. The results have shown that LuIII 3'-DsRed-3' and LuIII 3'-DsRed-5' are capable to increase the fluorescence of the cells over time, thus indicating that both recombinant plasmids were capable of self-replication. The results also suggest that LuIII 3'-DsRed-3' replicates more efficiently than LuIII 3'-DsRed-5'. This research suggests that in the design genetic vector systems based on the LuIII genome the presence of identical termini may be more efficient, such as those described and currently being studied for parvovirus AAV (Burger et al., 2005).

CHAPTER V

Conclusions

Two different viral expression vectors were designed in this investigation: LuIII 3'-DsRed-3’ and LuIII 3'-DsRed-5’. These two viral constructs has a coding sequence for a reporter gene that expresses a red fluorescent protein called DsRed. Both were transfected with a NS1 expression system in HeLa cells by electroporation, and then, monitored at different time points (12, 24, 48, 72, and 96 hours) post-transfection under a fluorescent confocal microscope.

All transfected HeLa cells showed expression of DsRed. LuIII 3'-DsRed-3’ and LuIII 3'-DsRed-5’ containing cells showed maximum fluorescence at 96 hours post-transfection, thus indicating that both plasmids were capable of self-replication. The results also suggest that LuIII 3'-DsRed-3’ has better replication efficiency than LuIII 3'-DsRed-5’ because more fluorescent HeLa cells were counted by Flow Cytometry in comparison to the positive control (pCMV-DsRed). Its important to point out that the positive control replicates as a plasmid while the LuIII constructs (LuIII 3'-DsRed-3’ and LuIII 3'-DsRed-5’) are expected to replicate as autonomous molecules that are initially excised by NS1 from the plasmids. These differences between the positive control (pCMV-DsRed) and the LuIII constructs (LuIII 3'-DsRed-3’ and LuIII 3'-DsRed-5’) in the nature of the molecules may contribute significantly to the differences in results observed by Fluorescent Microscopy and Flow cytometry In addition this research suggests that when a parvovirus vector is designed for gene

therapy the presence of identical termini may result in a more efficient vector. Such as those described and currently being studied for parvovirus AAV. Our data suggests that identical terminals in LuIII may be more efficient, as observed in our results (described in Figure 14 and Table 5).

CHAPTER VI

Recommendations

1. Repeat Flow Cytometry experiments with an instrument capable of counting more than ten thousand cells.
2. Design a plasmid containing two right end terminals to compare the replication efficiency against the minigenome 3'-DsRed-3’.
3. Insert of the AT-rich region (nt. 4558-4604) of LuIII into the minigenome 3’DsRed-3’ to compare the replication efficiency against a minigenome lacking this AT sequence.

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Appendices

Appendices

Appendix 1: Transformations of *E. coli* DH5α competent cells with Parvovirus LuIII Minigenomes.

Transformations (~15 ng DNA[a])	Number of colonies *E. coli DH5α* obtained Colony Forming Units				Transformation efficiency CFU/mL
	3'-DsRed-3' clone	3'-DsRed-5' clone	pCMV-DsRed clone	Standard control puc19	
1	4	0	42	334	2.5×10^5 CFU/µg
2	8	3	25	322	2.4×10^4 CFU/µg
3	0	0	35	195	1.4×10^3 CFU/µg
4	0	0	45	186	1.3×10^2 CFU/µg
5	3	2	14	365	2.7×10^5 CFU/µg
6	5	7	20	334	2.5×10^5 CFU/µg
7	0	6	35	263	1.9×10^3 CFU/µg
8	0	0	58	225	1.6×10^3 CFU/µg
9	2	0	17	458	3.4×10^6 CFU/µg
10	0	0	45	228	1.7×10^3 CFU/µg
11	0	15	25	322	2.4×10^4 CFU/µg
12	11	8	72	455	3.4×10^6 CFU/µg
13	13	0	63	420	3.1×10^6 CFU/µg
14	10	14	76	512	3.8×10^6 CFU/µg
15	13	9	68	450	3.3×10^6 CFU/µg

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