STUDY OF A POSSIBLE REPLICATION ORIGIN FUNCTION OF MAP UNITS 88-100 OF PARVOVIRUS LUIII IN HELA CELLS

By

Sara I Marí

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

BIOLOGY

UNIVERSITY OF PUERTO RICO

MAYAGÜEZ CAMPUS

2008

Approved by:

_____	_____
Juan Carlos Martínez Cruzado, PhD. Member, Graduate Comittee	Date
_____	_____
Carlos Ríos Velázquez, PhD. Member, Graduate Committee	Date
_____	_____
Nanette Diffoot-Carlo, Ph.D. President, Graduate Committee	Date
_____	_____
Julia O'Hallorans, Ph.D. Representative of Graduate Studies	Date
_____	_____
Nanette Diffoot-Carlo, PhD. Director of Biology Department	Date

Abstract

Studies to elucidate the mechanism of parvoviral replication are ongoing due to their potential as transient vectors for human gene therapy. Parvovirus LuIII, a single stranded DNA virus, has short double-stranded hairpin structures at the 3´and 5´ ends which serve as primers for replication. A replication origin at the left terminus has been characterized, yet a replication origin at the right terminus has not been identified. Homology of an A/T rich region at the 5' terminus of LuIII with the autonomously replicating sequences of yeast, suggests a replication origin function. To determine if the A/T sequence could function as an origin of replication in higher eukaryotic cells, a plasmid lacking an origin of replication and containing the reporter gene DsRed2 and the A/T sequence was constructed and transfected into HeLa cells. The results revealed a replication origin function for the LuIII A/T sequence.

Resumen

Los estudios para dilucidar el mecanismo de replicación de parvovirus están en curso debido a su potencial como vector transciente para la terapia génica humana. Parvovirus LuIII es un virus de ADN de hebra sencilla, sin embargo los terminales 3' y 5 ' se pliegan adquiriendo una forma de horquilla los cuales sirven como cebadores en la replicación. El terminal izquierdo del genoma de LuIII se ha caracterizado y se ha propuesto la presencia de un origen de replicación, sin embargo, en el terminal derecho no se ha identificado hasta el momento ningún origen de replicación. En el terminal 5´del genoma de LuIII se encuentra una región rica en A/ T la cual muestra una gran similitud con la secuencia de replicación autónoma presente en levaduras, sugiriendo que esta región podría funcionar como origen de replicación. Para determinar si esta secuencia rica en A/ T podría funcionar como un origen de replicación en células eucariotas superiores, se construyó un plásmido que contiene un gen reportero DsRed2 y carece de un origen de replicación, en su reemplazo se encuentra la secuencia rica en A/ T. Células HeLa fueron transfectadas con dicho plásmido. Los resultados sugieren que la secuencia rica en A/ T de LuIII aparenta tener una posible función como origen de replicación.

Dedication

A mi gran amor y compañero Claudio,
a mi adorable hija Zoe y a mi ángel Bautista.

Agradecimientos

Quiero agradecer ante todo a Dios por haberme dado la fortaleza para realizar este trabajo, por haberme puesto en mi camino a mucha gente la cual ha influido en una u otra manera en la realización de este trabajo. Quisiera agradecer a la Dra. Diffoot por haberme dado la maravillosa posibilidad de trabajar en su laboratorio de virología, por haber creído en mis destrezas y conocimientos. Gracias a mi comité, Dr. Carlos Ríos-Velázquez y Dr. Martínez Cruzado, por haberme guiado y ayudado en este trayecto; también quiero agradecer al Dr. Matías Cafaro, que además de un jefe fue un amigo.

Quiero agradecer a Naira Rodríguez, Pablo López y al Dr. Yamamura, por haberme permitido utilizar el “Flow Cytometer” de la Escuela de Medicina de Ponce.

Gracias a “mis compañeros de laboratorio”, mis confidentes, mis amigos, gracias por haber sido tan pacientes, he sido muy afortunada en haberlos tenido a mi lado. Gracias por esos momentos especiales que compartimos juntos, Ida, Mily, Omayra, Alina, Mildred, Lisandra, Alexa y Samir, gracias por tantos buenos y no tan buenos momentos, pero que me han dejado una marca en mi persona. Gracias a Mary y María, sin ustedes mi vida hubiese sido un poco más complicada.

A mi familia del Triatlón, a los que cariñosamente llamo “los chicos de Claudio” gracias por haberme hecho saber que podía, gracias por haberme dejado ser su guía, en realidad eso fueron ustedes para mí.

Finalmente, quiero darle las gracias a mi familia, que aunque nos separan unos cuantos kilómetros estuvieron siempre presentes. Má y Pá su amor incondicional, Lucas, mi hermano trotamundo, que me enseño que la vida hay que vivirla tal como se presenta GRACIAS infinitas.

Table of Contents

	Page
List of Tables	vii
List of Figures	viii
Chapter I: Introduction	1-2
Chapter II: Literature Review	3
a. General Characteristics	5
b. Classification	6
c. Viral Particle	10
d. Genome Structure	11
e. Parvovirus Genome Organization and Transcription	14
f. Parvovirus Encapsidation	20
g. Parvovirus Replication	23
i MVM Replication	24
ii LuIII Replication	29
h. LuIII A/T rich sequence	31
i. Objectives	35
Chapter III Materials and Methods	36
a. Plasmid Construction	36
i Construction of pDsRed CMV	36
ii Construction ofpDsRed CMV (-SV40)	40
iii Construction of pd2EGFP LuIII 88-100	42
iV Construction of pDsRed LuIII 88-100	45
b. Preparation of Competent cells	48
c. Transformation of Competent cells	48
d. Plasmid DNA extraction and Restriction characterization	49
e. Tissue Culture	50
f. Transfection Assay	50
g. Fluorescent Microscopy	51

h. Flow Cytometer 51
i. DNA Extraction from HeLa cells 52

Chapter IV Results and Discussion 53
a. Transformation of the recombinant clones 53
i pDsRed CMV clone 54
ii pDsRed CMV (-SV40) clone 54
iii pDsRed LuIII 88-100 clone 54
b. Restriction analysis of recombinant clones 55
i pDsRed CMV recombinant clone 55
ii pDsRed CMV (-SV40) recombinant clone 57
iii pDsRed LuIII 88-100 recombinant clone 59
c. Transfection of HeLa cells and Observation in Microscope 61
d. Quantitative Analysis by Flow Cytometry 67
e. Extraction of recombinant molecules, pDsRed CMV and pDsRed LuIII 88-100, from HeLa cells, and their Bacterial Transformation into *E. coli DH5α* 72
f. Restriction Analysis of Recovered Recombinant Plasmid 77

Chapter V Conclusion 80
Chapter VI Recommendations 81
Literature Cited 82

List of Tables

Tables		Page
Table 1	Classification of *Parvoviridae* family as established by the ICTV	7
Table 2	Encapsidation Pattern of Selected Parvovirus	20
Table 3	Transformation of *E. coli DH5* with the products from the different ligations	49
Table 4	Percentage of fluorescent HeLa cells after transfection with plasmids indicated; schematic representation of percentage of fluorescent cells	71
Table 5	*E coli DH5α* transformants with possible pDsRed CMV, PDsRed CMV (-SV40), and pDsRed LuIII 88-100 clones obtained from the transfected cells	76

List of Figures

Figures		Page
Figure 1	Palindromic Termini of Parvovirus LuIII.	13
Figure 2	LuIII and MVM Parvovirus genome organization	14
Figure 3	Structure of LuIII Parvovirus Genome.	16
Figure 4	Minute virus of mice (MVM) transcriptional map	17
Figure 5	Transcriptional map of AAV 2	19
Figure 6	Modified rolling hairpin model for MVM DNA replication	26
Figure 7	The dimer junction resolution and minimal origin of replication	28
Figure 8	Proposed Model for the Replication of Parvovirus LuIII	30
Figure 9	Modular structure of ARS1 in *Sacharomyces cerevisia*e	33
Figure 10	Construction of pDsRed2-1CMV (positive control)	38
Figure 11	Digestion of pd2EGFP-1 CMV and pDsRed2-1 with the restriction enzymes *Eco*R I and *Bam*H I	39
Figure 12	Construction of pDsRed2-1 CMV (-SV40) (negative control)	40
Figure 13	Digestion of pDsRed-CMV with *Cla* I- *Ssp* I	41
Figure 14	Construction of pd2EGFP LuIII 88-100	43
Figure 15	Digestion of pd2EGFP and pGLu883ΔXba with *Eco*R I-*Xba* I	44
Figure 16	Construction of the pDsRed LuIII 88-100	46
Figure 17	Digestion of pd2EGFP LuIII 88-100 and pDsRed CMV	47
Figure 18	Restriction analysis of pDsRed CMV (positive control)	56
Figure 19	Restriction analysis of pDsRed CMV (-SV40) (negative control)	58
Figure 20	Restriction analysis of pDsRed LuIII88-100	60
Figure 21	Non transfected HeLa cells (controls)	62
Figure 22	HeLa cells transfected with pDsRed CMV (positive control)	64
Figure 23	HeLa cells transfected with pDsRed-LuIII 88-100	65
Figure 24	HeLa cells transfected with pDsRed CMV (-SV40) (negative	66

control)
Figure 25 Spatial distribution of cells when the flow cytometer is used 68
Figure 26 Spatial distribution of cell population after 72 hrs post-transfection 70
Figure 27 Observation of HeLa cells transfected with pDsRed CMV, pDsRed CMV (-SV40) amd pDsRed LuIII 88-100 at 72 hrs after being passaged 74
Figure 28 Restriction analysis of transformants colonies of Positive control and pDsRedLuIII88-100 obtained from HeLa cells 78

CHAPTER I

Introduction

Through years of research, it has been established that parvoviruses could be a valuable tool for gene therapy (Faisst and Rommelaere, 2000). Parvoviruses are some of the smallest viruses found in nature (hence the name, from latin parvus meaning small). These viruses are highly dependent on host cell functions for a productive infection. Their replication only occurs in the nucleus of permissive cells during S-phase, suggesting the need for specific cellular replication machinery in the viral replication process (Berns, 1990). Parvovirus, commonly called parvo, is a genus of the Parvoviridae family, linear, non-segmented single stranded DNA viruses with an average genome size of 5 Kb.

The single-stranded DNA encapsidated by parvoviruses can have 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 (complementary to the mRNA), therefore the autonomous parvovirus LuIII encapsidates both DNA strands with equal frequency (Bates *et al.,* 1984).

The termini of their genome contain palindromic sequences that fold into stable double stranded hairpin-like structures and serves as a primer for the LuIII replication (Rhode *et al.,* 1997). 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 (Astell et al., 1985; Cotmore and Tattersal, 1995; Tam and Astell, 1993).

Diffoot and co-workers (2005) proposed a model where the synthesis of the minus strand requires replication from the left palindrome of the plus strand DNA, whereas the plus strand synthesis requires replication from the right termini of the minus strand. Comparison between the parvovirus MVM, H-1 and LuIII entire genome revealed an 80% sequence homology; the difference is a 47 bp A/T-rich sequence at nucleotide 4558 (m.u. 89) present at the end of the right open reading frame that is unique for LuIII virus.

It has been postulated that this sequence is responsible for the synthesis of the plus strand, playing an important role during LuIII replication and encapsidation. Recent data (Arroyo, 2000) suggest that this AT-rich (47 bp) sequence can act as an autonomously replication sequence (ARS) as those found in yeast, but in a weakly way. Generally, replication origins contain internal repeats that are rich in adenine-thymine base pairs. This data suggest that the A/T rich region of LuIII alone cannot act as an efficient autonomously replicating sequence; this region requires additional LuIII sequences present in the right terminus (m.u. 88-100) to enhance the observed ARS function in yeast. De Jesús (2004) constructed a plasmid that contained map unit 88-100 of LuIII and demostrated that this sequence can act as a replication origin in yeast (De Jesús-Maldonado, 2004).

To elucidate the possible function of LuIII A/T rich region as a replication origin, a plasmid containing the m.u 88 - 100 of LuIII genome region was constructed, pDsRed LuIII 88-100. HeLa cells were transfected with this plasmid and observed under a fluorescent microscope. The data suggest that the mu 88-100 containing the A/T rich region can function as a replication origin.

CHAPTER II

Literature Review

Gene therapy is a technique used to correct defective genes responsible for disease development. The most popular approach is the insertion of a normal gene into the genome to replace an abnormal gene that causes the disease. This technique involves transfer of genetic material to target cells using a delivery system, or vector. The delivery of therapeutic genes to particular cells poses the greatest challenge to the successful application of gene therapy in humans. Considerable effort is being put into the development of more selective and efficient vectors. They target specific cell populations or contain promoters that are selectively activated in certain cells.

Viral and nonviral vector systems for gene delivery are available. Currently, the most common vector is a virus that has been genetically altered to carry normal human DNA. Some viruses enter the cells by receptor-mediated processes, escape from endosomes and introduce their genetic material into the host cell nuclei as part of their replication cycle. Several types of viruses are in use, or are being developed as gene therapy vectors. The interest has centered on four types of viruses. Among them are Herpes Simplex Viruses, Retroviruses (including lentiviruses), Adenoviruses and Adeno-associated viruses (Strachan and Read, 1999).

Herpes simplex Virus (HSV) are trophic for the central nervous system (CNS). Their major expected applications involve the delivery of genes into neurons for the treatment of neurological diseases, such as Parkinson's disease, and for treating CNS tumors (Strachan and Read, 1999).

The Lentivirus family, which includes HIV (human immunodeficiency virus) are able to transduce dividing or nondividing cells. Because of their ability to infect nondividing cells and to integrate into the host cell chromosomes, considerable efforts are now being devoted to making lentivirus vectors (Strachan and Read, 1999).

Adenoviruses are DNA viruses that have a natural tropism for respiratory epithelium, the cornea, and the gastrointestinal tract. Adenovirus vectors are one of the most popular delivery systems in gene therapy with extensive applications in this area for cystic fibrosis and certain types of cancer. They are human viruses which can be produced at very high titers in culture, and they are able to infect a large number of different human cell types including nondividing cells. Entry into cells occurs by receptor-mediated endocytosis and transduction efficiency is very high (often approaching 100% *in vitro*). They are large viruses and so have the potential for accepting large inserts (Strachan and Read, 1999).

Adeno-associated viruses (AAVs) usually cannot undergo productive infection without co-infection by a helper virus, such as an adenovirus or herpes simplex virus. They have a broad tissue tropism (i.e., lung, muscle, central nervous system, liver, and retina) as a consequence of attachment of Adeno-associated viruses type 2 (AAV-2) to the ubiquitously expressed cell surface heparan sulfate proteoglycan (HSPG) protein (Kufe, *et al.,* 2000). Recombinant AAV vectors, rAAVs, transduce both replicating and nonreplicating cells. This type of virus is being used, because it is non-pathogenic (most people carry this harmless virus). However, most people treated with AAV will not build an immune response to remove the virus and the cells that have been successfully treated with it. They have the advantage of providing a long-term gene expression, and chromosomal DNA integration, at a specific site on 19q13.3-qter without causing oncogenicity or mutagenesis (Rabinowitz and Samulski, 2000).

AAV seems like an appropriate candidate for long-term gene therapy against genetic defects and cancer. However, in some situations, long-term persistence may be undesirable and there is a need for a small, non-integrating viral vector. Viruses used for gene therapy have some disadvantages such as: (a) patient immune recognition of viral proteins with a consequent immune attack and destruction of infected cells, (b) restriction in size and quantity of transgene, (c) inability of repeated viral administration. Most clinical studies have been carried out using retroviruses or adenoviruses as transfer vectors with various advantages and disadvantages. The targets of viral therapy

can be: inactivation of oncogenes (ras, c-myc), genes involved in tumor progression (Triple- helix formation) and tumor associated antigen genes. Some of the autonomous replicating parvoviruses, such as H-1, MVM and LuIII, have properties that make them interesting candidates for gene therapy. For instance, they do not integrate their DNA into the host chromosomes, they result in lytic or persistent infections, they are nonpathogenic to humans but can infect human cells, and they are oncotrophic and oncolytic in transformed cells. They have a strong predilection for tumor cells (Corsini *et al.,* 1996; Cornelis et al., 2004), and they do not require a helper virus. They are strictly dependent on S phase of the cellular cycle (Blechacz and Russell, 2004).

A therapeutic approach, using autonomous parvoviruses, implies designing recombinant parvovirus-based vectors which (1) can be efficiently packaged into infectious viral particles, (2) retain the parvoviral tropism for tumor cells, and (3) selectively express the therapeutic gene (Dupont *et al*., 2000 mentioned in Wong, J.R. and C. Saswati. 2002; Gancberg *et al*., 2000; Haag *et al*., 2000). The possible use of vectors based on the autonomous parvovirus LuIII for use in cancer therapy and in general the potential use for therapeutic purposes can only be accomplished by elucidating the basic biology of this parvovirus (Maxwell *et al.*, 2002).

General Characteristics

The parvoviridae family includes one of the smallest known viruses, and some of the most environmentally resistant. They were discovered during the 1960's. They infect vertebrates and insects, are widespread in nature, and include a broad variety of viruses that replicate in the nuclei of hosts, ranging from insects to humans (Cotmore and Tattersall, 1987; Siegl, G. 1984).

Parvoviridae have a genome consisting of a linear single-stranded DNA and an icosahedral capsid. They are small, measuring only 18-26 nm in diameter. They can only replicate in dividing cells. The virus itself is very stable, surviving temperatures of up to 60 degrees Celsius at a pH ranging from 3 to 9 (Maxwell *et al*., 2002).

Classification

According to the International Committee on Taxonomy of Viruses, the *Parvoviridae* family is divided into two groups, based on their host range and whether or not they depend on helper functions of other viruses to replicate. The viruses which are dependent on a helper virus for replication are named Defective viruses; viruses which are competent for replication, are called Autonomous viruses.

Taxonomical classification of this family is divided in two subfamilies: *Densovirinae,* viruses which can replicate in insect host cells or other arthropods, and *Parvovirinae* viruses that can infect a range of vertebrate species (Table 1) (Van Regenmortel *et al.*, 2000).

Table 1: Classification of *Parvoviridae* family as established by the ICTV*.

Subfamily	Genus	Species
Densovirinae	*Densovitus*	*Junonia coenia densovirus*
	Iteravirus	*Bombyx mori densovirus*
	Brevidensovirus	*Aedes aegypti densovirus*
	Pefudensovirus	*Periplanta fuliginosa densovirus*
Parvovirinae	*Erythrovirus*	*Human parvovirus B19*
		Simian parvovirus (SPV)
	Dependovirus	*Adeno-associated virus 1-6 (AAV)*
	Amdovirus	*Aleutian mink disease virus*
	Bocavirus	*Bovine parvovirus (BPV)*
		Minute Virus of Mice (MVM)
	Parvovirus	*lapine parvovirus*
		LuIII parvovirus
		Feline parvovirus
		H-1 parvovirus

*ICTV = International Committee on Taxonomy of Viruses. http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/index.htm

The ***Densovirinae*** **subfamily** is composed of viruses that replicate autonomously, and infect invertebrates, including economically or medically important insect groups, such as Lepidoptera, Diptera, Orthoptera, Dyctioptera and Odonata. These viruses are known as Densonucleoviruses (DNVs), because the diseases are characterized by the accumulation of viral particles in the nuclear compartment, densonucleoses (Dumas, 1992). These viruses are highly pathogenic to their hosts, causing lethal diseases in natural or laboratory insect populations. Because of their pathogenicity on economically and medically important insects they are currently being studied, and also for their potential use as a tool in biological control of pests as well as for their possible use as gene transfer vehicles (Bossin *et al*., 2003).

Members of the ***Parvovirinae*** **subfamily** have been studied for their applications as vectors, particularly in gene therapy, ranging from long-term gene replacement to short-term expression. The *Parvovirinae* sub-family is divided into five genera, *Erythrovirus, Dependovirus, Amdovirus, Bocavirus* and *Parvovirus*. Even though all members of these genera are similar in structure, they can be separated by differences in host range, pathogenesis, clinical symptoms, gene expression and replication (Berns, 1996; Faisst and Rommelaere, 2000). Only members of the *Erythrovirus, Dependovirus* and *Bocavirus* genera are known to infect humans. Members of the Parvovirus genus like Minute Virus of Mice (MVM) and LuIII are best known for being autonomous parvovirus that infects mammalian cells.

The **genus *Erythrovirus*** is named for its tropism for red blood progenitor cells. This includes human parvovirus B19, the primate virus, SPV (simian parvovirus from Cynomolgus monkeys), and V9 (Cossart *et al.*, 1975).

Of all parvoviruses, B19 is the only one known to be a native human pathogen (Woolf *et al*., 1989). It is associated with the common childhood disease, erythema infectiosum or fifth disease, where up to 80% of adults in the population are infected (Heegard and Hornsleth, 1995; Lukashov and Goudsmit, 2001).

***Dependovirus* genus**, consisting of adeno-associated viruses (AAV), is naturally defective and requires a coinfection with a helper virus, usually adenovirus, to proliferate. Due to its inability to replicate in the absence of adenovirus, this defective parvovirus was named adeno-associated virus.

These viruses are capable of infecting both dividing and non dividing cells, and in the absence of a helper virus, integrate into a specific point in the host genome (19q 13-qter) (Kotin *et al.*, 1990; Berns and Linden, 1995; Vincent *et al*., 1997).

By phylogenetic analysis, two new genera, *Amdovirus* and *Bocavirus*, within the subfamily of *Parvovirinae* were recently created (International Committee on Taxonomy of Viruses Virus Index Database, ICTVdB www.danforthcenter.org_iltab_ictvnet). All members are autonomously replicating viruses that were earlier classified in the *Parvovirus* genus.

The major distinguishing feature of ***Amdovirus***, Aleutian mink disease virus, is the VP1 N-terminus, which is shorter than those of other parvoviruses, and, in contrast to that of most other parvoviruses, lacks a phospholipase A2 enzymatic core (Tattersall *P.,* 2005 mentioned in Zadori *et al.,* 2005), 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 mentioned in Qiu *et al.,* 2007). All members of Parvoviridae subfamily, have two major ORFs, however *bocavirus* also has a third middle ORF, which encodes a nonstructural nuclear phosphoprotein NP1 with an unknown function (s) (Qiu *et al.,* 2007).

***Parvovirus* genus** is capable of infecting a variety of mammalian cell species. They are termed autonomous viruses because they have all the information and functions necessary to replicate in host cells (Berns, 1996), but they require proliferating cells (S-phase functions) or early G2 phase and, in some cases, tissue-specific factors (Maxwell *et al.*, 2002). There is no evidence that the Autonomous Parvovirus (APVs) integrate in the cell genome during infection (Berns, 1996).

There are a variety of parvovirus sub-groups. Minute virus of mice (MVM), H-1 and LuIII are classified as rodent parvovirus. MVM was isolated from a stock of mouse adenovirus, H-1 was originally isolated as a contaminant of a human tumor cell line (Hep1) (Berns, 1996; Lukashov and Goudsmit 2001), and LuIII was isolated as a contaminant of a human lung carcinoma (Soike *et al*., 1976; Diffoot *et al*., 1989).

Viral Particle

Parvoviruses are among the smallest eukaryotic viruses known. The particles consist of non-enveloped icosahedral capsids that contain 50% of protein and 50% of DNA genome. There are 3 capsid proteins, VP1, VP2, and VP3. VP3 is derived by protease cleavage of VP2. The three-capsid proteins confer stability to the virions, which are resistant to inactivation by pH, solvents such as alcohol and ether and temperatures as high as 50℃ (Berns, 1996). Inactivation may be achieved by formalin, β-propiolactone and gamma irradiation (Faisst and Rommelaere, 2000).

The high stability of the virus capsid has epidemiological importance since it ensures that the virus can persist over long periods of time, once introduced in the population. The different autonomous parvoviruses cause a variety of heterogeneous diseases in their hosts with different organs and tissue involvement (tropism). Cell tropism is broad, but since cells must pass through S-phase, the viruses tend to infect rapidly dividing tissue (Caillet-Fauquet *et al.*, 1990).

The differences in the capsid surface are what determines tissue tropism, pathogenicity, and antigenicity among different parvoviruses and among the strains of a particular virus (McKenna, 1999).

Genome Structure

The most characteristic feature of parvoviruses is their genome structure, consisting of an approximately 5 kb single-stranded DNA strand. This single stranded DNA can be of positive (+) or negative (-) polarity (Astell *et al.*, 1985; Tam and Astell, 1993). The linear DNA molecule terminating in palindromic sequences, which are folded back on themselves to form hairpin-like structures by self-hydrogen bonding are very stable. The "hairpin" structures are critical for genome replication because these contain most of the *cis*-acting signals necessary for viral DNA replication and encapsidation (Astell *et al.*, 1985; Tam and Astell, 1993; Cotmore and Tattersal, 1995). The sequence and secondary structure of 3' and 5' termini can be different within individual genomes and species.

The adeno-associated viruses (AAV) genome is a single-stranded DNA, either positive- or negative-sensed with an inverted terminal repetition (ITR), which is present at both of its termini (Lusby *et al.*, 1980; Labow *et al.*, 1987). B19 also has identical terminal repeats (Berns, 1996). ITRs are the minimal essential cis-acting sequences required for viral replication, host integration, host excision and encapsidation (Lusby *et al*., 1980; Srivastava *et al*., 1983). The terminal repetition is 145 base pair long in which the first 125 nucleotides can self-base pair to form a T-shaped hairpin structure. The ITRs were named so because of their symmetry, which was shown to be required for efficient multiplication of the AAV genome. These sequences have the ability to form a hairpin, which contributes to the so called self-priming that allows primase-independent synthesis of the second DNA strand. The ITRs were also shown to be required for both integration into the host cell genome and rescue from it (Zhou and Muzyczka, 1998).

In contrast to the genomes of AAV and B-19, the 3' and 5' hairpins of most of the Autonomous Parvovirus, APVs, differ both in size and sequence (Berns, 1996).The autonomous parvoviruses also have hairpins at both 3' and 5' ends of their genome, but they are not an inverted terminal repetition, or a palindromic repetition. Minute Virus of Mice (MVM) and LuIII have different terminal palindromes; therefore employ different

resolution strategies for each terminus (Astel *et al*., 1985). The 3' terminus (left end of the minus strand) is 115 to 122 bases long, and can assume only the T-shape or Y-shape conformation, conventionally known as a rabbit ear conformation. The 5’ terminus (right end of the minus strand) is 200 to 211 bases long and can assume a T-shape or U-shape structure (Diffoot *et al*., 1993; Cotmore and Tattersal, 1995).

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

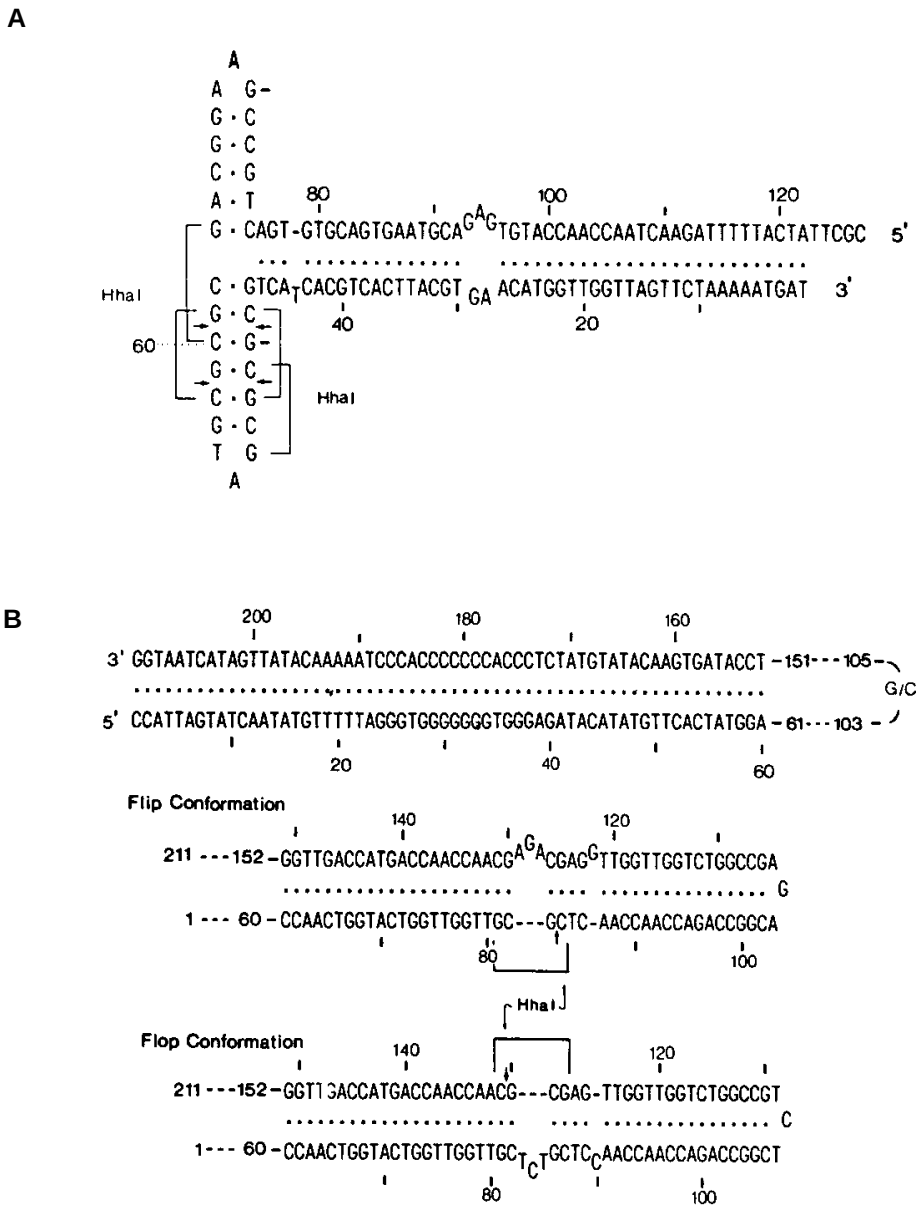


Figure 1: Palindromic Termini of Parvovirus LuIII. A: DNA sequence of the left terminus (3'end) of the minus strand of LuIII forming its characteristic T-shape secondary structure and flip conformation. **B**. DNA sequence of the right terminus (5'end) of the plus strand of LuIII forming the U-shape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

Parvovirus Genome Organization and Transcription

In general, the parvovirus genome is organized into two open reading frames (ORF) (Figure 2). One ORF extends from map unit (m.u.) 5 to 40, while the second one extends from m.u. 50 to 90. The transcription of these ORFs proceeds from left to right direction and starts at both promoters. Using this orientation, the left hand of the DNA genome encodes for replication, Rep, and non-structural, NS, proteins while the right hand encodes for structural or capsid proteins VPs. The pattern of gene expression varies depending on the number of promoters (1- 3 promoters) (Berns 1996; Diffoot *et al*., 1989). Although, dependoviruses and autonomous parvovirus have different life cycles, both their structural and non structural proteins have similar functions.

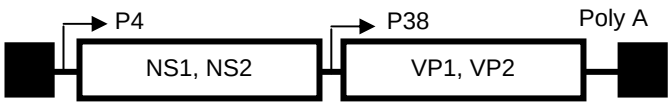


Figure 2: LuIII and MVM Parvovirus genome organization. Arrows indicate the promoters P4 and P 38; Black square: non identical terminal inverted repetitions.

The autonomous parvovirus MVM and LuIII are organized in two large open reading frames. Transcription starts at promoter P4, located at map units 4, and P38, at m.u 38, from left to right respectively (Figure 3 and 4) (Diffoot *et al.*, 1993)

In general, the early promoter (**P4**) controls the transcription of the non-structural proteins NS1 (71-83 kDa) and NS2 (24-28 kDa) (Cotmore and Tattersall, 1990; Cotmore and Tattersall 1994). NS1 is the most important non-structural protein, essential for productive replication in all cell types; it coordinates the progression of the parvoviral

replication fork. NS1 is a site-specific duplex DNA binding protein and also has single-strand nickase activity (Naeger *et al*., 1993; Christensen and Tatersall, 2002). The small non-structural protein NS2 consists of three isoforms (NS2-P, -L, and –Y) that differ at their carboxy termini as a result of alternative splicing events. NS2 protein is mainly located in the cytoplasm and share a common amino-terminal domain with NS1, which comprises the first 85 amino acids of each protein (Cotmore and Tattersal, 1995). This protein exists in phosphorylated and unphosphorylated forms that are mainly located in the cytoplasm of infected cells; however, nonphosphorylated NS2 can also be found in the nucleous. The role of NS2 is still elusive; it has been previously reported to play a critical role in viral capsid assembly and consequently in the generation of viral single-stranded DNA, and in viral mRNA translation (Cotmorem *et al.*, 1997; Eichwald *et al*., 2002).

The right ORF, (**P38**), drives the synthesis of the capsid polypeptides VP1 (80-86 kDa), VP2 (64-75 kDa) and VP3 (60-62 kDa) (Shade *et al.*, 1986; Cotmore and Tattersall. 1987; Berns, 1996). The major capsid protein is VP2, by itself, is capable of virion formation and DNA packaging. However, the minor protein, VP1, is required for infectivity (Tullis *et al*., 1993; Maxwell *et al*., 1995). While VP1 and VP2 share most of their sequence, VP1 has a unique N-terminal region of approximately 140 amino acids which 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). The third capsid protein (VP3) is not a primary translation product, but is derived by proteolytic cleavage which removes the amino-terminal region of VP2 and which occurs only after capsid assembly and packaging of the viral genome (Berns, 1996).

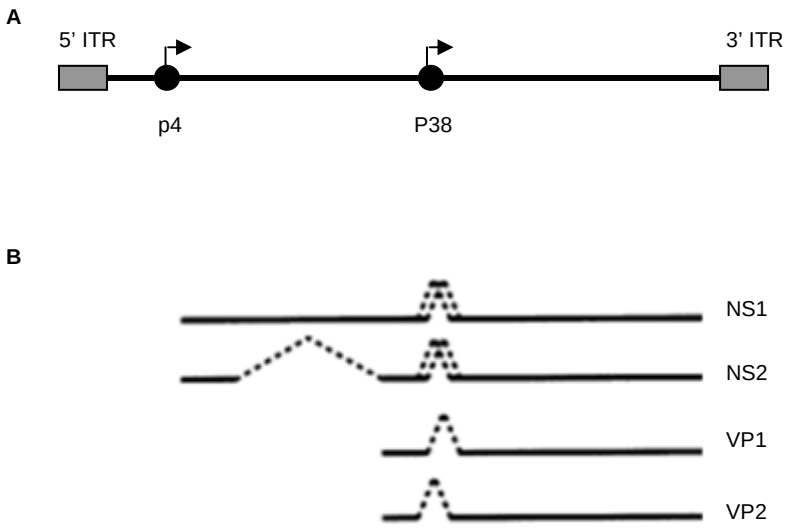


Figure 3: Structure of LuIII Parvovirus Genome. A: Genome organization. **B:** Transcripts. Transcription initiates from two promoters P4 and P38 shown by arrows and terminates at a common poly A signal. Alternative splicing is shown by doted line. The P4 and the P38 transcripts generates mRNAs encoding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

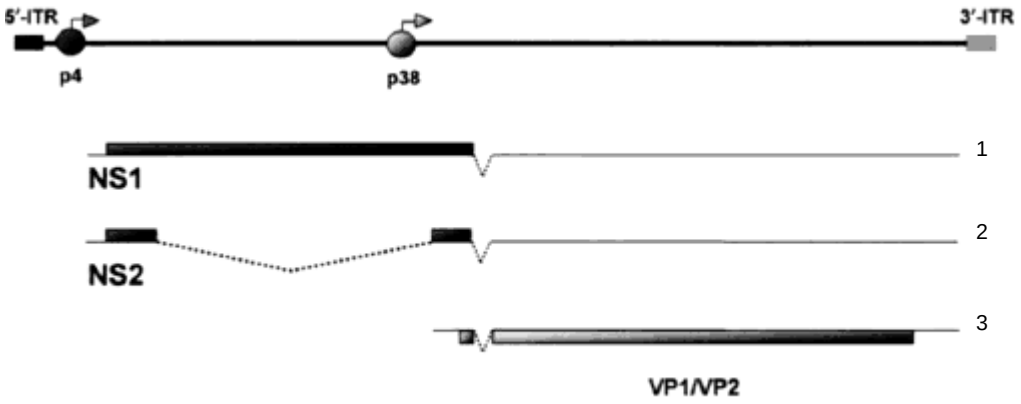


Figure 4: Minute virus of mice (MVM) transcriptional map. The genome is organized into two open reading frames (ORF). The location of the two promoters P4 and P38 is shown by arrows. The ITR's are shown by boxes at both sites. 1 is showing the major transcript class that encodes for the non-structural 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)

Unlike the autonomous parvovirus, the dependovirus AAV genome comprises three promoters (P5, P19 and P40) that drive the synthesis of mRNAs coding for Rep and Cap proteins, named for their role in DNA replication and encapsidation respectively. A polyadenylation signal is common to all transcripts. The synthesis of mRNAs coding for 4 Rep-proteins (Rep78, Rep68, Rep52 and Rep40) are generated from the P5 and P19 promoter. These proteins are equivalent to the NS proteins of other parvoviruses. Those proteins differ in their C-termini due to alternative splicing of the transcripts. The Cap ORF encodes three structural proteins (VP1, VP2 and VP3) generated from transcripts initiated from P40 promoter (Berns 1991; Kyostio *et al*., 1994).

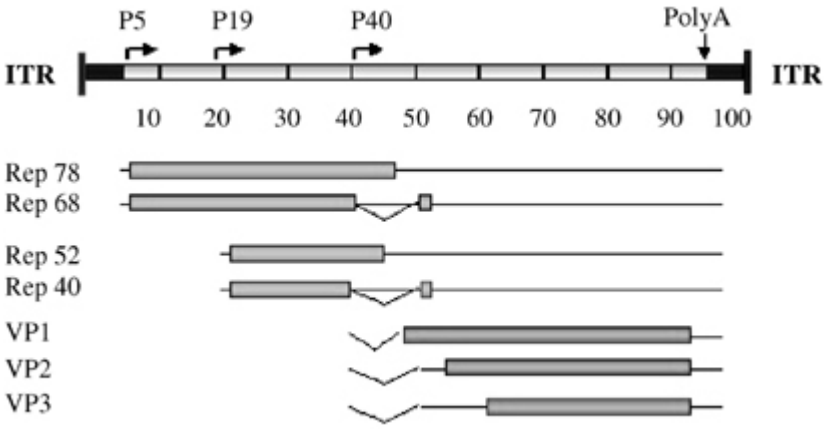


Figure 5: Transcriptional map of AAV 2. The genome is organized into two open reading frames. The terminal repetitions are represented as small boxes. The location of the three AAV transcription promoters, P5, P19 and P 40, as well as the common polyadenylation site are indicated by arrows.

Parvoviral Encapsidation

Parvoviruses encapsidate a linear single-stranded DNA. They can either package equal amounts of plus and minus strands or mostly minus strand DNA. It has been established that the *cis*-elements required for packaging DNA reside in the parvoviral genomic termini (Muller and Siegl, 1983; Cotmore and Tattersall, 2005).

The parvoviruses, minute virus of mice (MVM) and H-1 virus, encapsidate predominantly the minus strand of the genome (Bates *et al.*, 1984; Chen *et al*., 1986). Other parvoviruses, such as adeno-associated virus, LuIII, and B19 virus, efficiently encapsidate either strand of the genome (Berns, 1990) (Table 2).

Table 2: Encapsidation Pattern of Selected Parvovirus

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

The mechanisms used for the strand-packaging are not understood, but the available data suggests that nascent genomes are inserted into preformed capsids (Muller and Siegl, 1983; Cotmore and Tattersall, 2005). 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 (99%) the minus strand (Astell *et al*., 1983; Astell *et al*., 1986). Current evidence, however, indicates that uniqueness of the termini per se is not the

determinant of packaging. In particular, LuIII encapsidates approximately equal amounts of both strands and its terminals are different (Bates *et al*. 1984; Diffoot *et al*., 1989).

The mechanism by which LuIII encapsidates either strand of its genome remains unknown. Willwand and his colleagues (1993) have shown 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. In view of this, it is possible that LuIII contains recognition sequences in both termini, while MVM, H-1 virus, and others contain these sequences in only their left terminus. However, other explanations exist. Alignment of LuIII with the MVMp genome shows that LuIII has more than 80% identity with the rodent parvoviruses MVM and H-1 virus. In LuIII, an insertion exists with respect to the MVM genome. This sequence is an AT-rich stretch of 47 nucleotides, (TATGCTCTATGCTTCATATATATTATATATATTATTATACTAACTAA) beginning at nucleotide 4560 of the LuIII genome. It was hypothesized that this sequence, termed the AT-rich sequence, might be responsible for the ability of LuIII to package both strands of its genome (Diffoot *et al*., 1993; Corsini *et al.*, 1995).

Tam and Astell (1993) have identified a sequence near the right palindrome of MVM as a *cis*-acting replication signal. This region has been shown to bind four cellular proteins which are thought to be involved with replication of the MVM genome. It is possible that the 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. If this is the case, 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. Thus, disruption of this protein-binding region by AT might eliminate strand-selective encapsidation and confer the ability to encapsidate either strand (Corsini *et al*., 1995).

A study done with a chimeric virus of the MVM genome, MML, in which >95% MVM sequence was fused to the right-hand terminus of LuIII, packages >40% positive-sense DNA. This virus attempts to encapsidate both positive- and negative-sense genomic sequences, but encounters difficulty in internalizing specific elements in the plus strand. Successfully packaged full-length positive-sense MML DNAs were found to be rare, whereas partially packaged genomes, of plus strand, accumulated to high levels in infected cells. This suggests that the negative-strand sequence of MVM has been under selective pressure to optimize its compatibility with the encapsidation mechanism, whereas the positive-strand sequence has not (Cotmore and Tattersall, 2005b). 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 and reiterated G4 clusters, thus, ensuring optimal compatibility with the translocation machinery. In viruses from the Amdovirus, Parvovirus, and Bocavirus genera (Tattersall *et al*., 2005 cited by Cotmore and Tattersall, 2005b), which package predominantly the negative strand, stretches of G4 or longer are entirely absent from the minus strand compared to the plus strand. Thus, for example, the negative-sense single-stranded regions of AMDV, MVM, H1, KRV, FPV, and BPV are entirely devoid of such G tetrads, whereas their equivalent positive-sense sequences contain 20, 14, 15, 19, 21, and 49 G4 clusters, respectively. In contrast, for viruses from the Dependovirus and Erythrovirus genera, which package either strand with equal efficiency, such runs of G are equally represented on both strands; therefore, the packaged strand sequence is under selective pressure at the structural level (Cotmore and Tattersall, 2005).

Another study 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). Accordingly, 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. Their data analyses indicated that the packaging

substrate is the newly released single strand and not the duplex RF forms of the genome. They also proposed 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 RF during the packaging phase of infection. Hence, the plus strands are only released if the right end nick site is suboptimal, making the displaced minus strands cycle through an obligatory dimer duplex intermediate (Cotmore and Tattersall, 2005).

Viruses package their genomes into protein capsids either via association of structural proteins with the viral genomes or via insertion of viral genomes into preassembled capsids (Kings *et al*., 2001). Parvovirus initiator protein is left covalently attached to the 5' end of the nicked DNA after replication (Im and Muzyczka, 1990; Prasad and Trempe, 1995). Therefore, the genomes are marked for packaging by their association with the non structural proteins, which in turn, bind efficiently to intact empty capsids. Still the underlying mechanism of how the empty capsids are recruited to newly displaced single strands remains unknown (Cotmore and Tattersall, 2005b).

Parvovirus Replication

Parvovirus replication and assembly occurs in the nucleus, is dependent of host cellular functions and occurs only in dividing cells, with the use of cellular enzymes. The mechanism of replication of the genome is unique to this virus family.

The replication is activated by NS1 binding to the duplex recognition sequence, (ACCA)*n*, present in all viral origins, and introducing a strand-specific nick at an adjacent initiation site. This transesterification reaction creates a base-paired 3' nucleotide to serve as a primer for new DNA synthesis while leaving NS1 covalently attached to the 5' end of the DNA at the nick site, where it likely serves as a 3' to 5' helicase (Cotmore and Tattersall, 2003).

The viral replication strategy resembles rolling circle replication (RCR) using unidirectional leading-strand DNA synthesis and probably evolved from prokaryotic RCR

replicons. Replication initially proceeds rightward from left (3') to right (5'). 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 (RF) (Diffoot *et al.*, 2005). These hairpins serve as "hinges" that repeatedly unfold and refold during replication, reversing the direction of synthesis at each end of the linear molecule and creating long palindromic replicative-form.

- **MVM replication**

MVM has short palindromic sequences at both DNA termini that fold into stable hairpin for initiation of synthesis of plus sense DNA, resulting in double stranded-DNA. The hairpin structure is then again used as a primer to transcribe more minus-sense strands from the ds DNA. The current model for the mechanism of viral replication postulates that the growing strand replicates back on itself, producing a tetrameric form from which two plus strands and two minus strands are generated by endonuclease cleavage. (Berns, 1990; Burnett *et al*., 2006; Astell, 1986).

In the viral single strand, the 3' hairpin assumes a Y-shaped structure in which there are two internal hairpins, forming the ears, and a third palindrome forming a stem. Within the stem there is an important mismatched "bubble" sequence where a triplet, 5'GAA3', on one strand opposes a dinucleotide, 5'GA3', on the other, only the strand containing the GA dinucleotide functions as a replication origin (Cotmore and Tattersall, 1994). Potential origin sequences cannot be nicked by NS1 in this hairpin configuration. Instead, rolling-hairpin displacement synthesis must proceed, so that the hairpin is unfolded and copied to create the fully base-paired palindromic junction, spanning adjacent genomes in dimer replicative form, before the active origin is generated (Burnett *et al*., 2006).

The MVM DNA replication involves successive steps depicted schematically in Figure 6 (Kuntz-Simon *et al*., 1999). The single-stranded virion DNA gets converted into

a closed, double-stranded, monomeric replicative form (cRF) by extension of the left terminal hairpin (3') and ligation of the growing strand to the folded-back right terminal hairpin (5') (step 1) (Cotmore *et al*., 1989; Baldauf *et al*., 1997). Further processing of cRF DNA requires the activity of the viral nonstructural protein NS1 (Cotmore and Tattersall, 1998; Cotmore *et al*., 2000).

NS1 does a sequence-specific nick at the cRF right telomere, followed by initiation of displacement synthesis and terminal extension, giving rise to an extended molecule (5'-terminally extended monomeric replicative form 5'eRF (step 2). Hairpin refolding at the extended terminus, supported by host cell nuclear factors and efficiently stimulated by NS1, creates a so-called rabbit-ear structure (5'-rabbit-eared monomeric replicative form 5'reRF (step 3). This structure provides a primer for strand displacement and synthesis of the dimeric replicative- form (dRF) formation (step 4). After being replicated to the dimer intermediate, the extended hairpin forms a palindromic double-stranded sequence termed the dimer junction, because it bridges two unit lengths head-to-head duplex genomes. In the dimer junction, the nucleotides of the bubble sequence now occur as duplex DNA, on each side of an axis of symmetry, creating the GAA and TC arms, where the two potential origin sequences differ in length by a single nucleotide. During replication, the dimer junction is resolved into two structures by NS1. The extra nucleotide in the GAA arm renders this origin inactive and restricts replication initiation to the TC arm of the dimer junction, causing the resolution process to be predominantly asymmetric. The two types of monomeric replicative form (RF) DNA are covalently closed (5'eRF) or extended (3'-5'eRF) left-hand termini, respectively (steps 5a and 5b). The 5'eRF molecule generated in this way reenters the cycle as in step 3, while duplex-to-hairpin transition at the right-hand palindrome of the 3'-5'eRF molecule (step 6) 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).

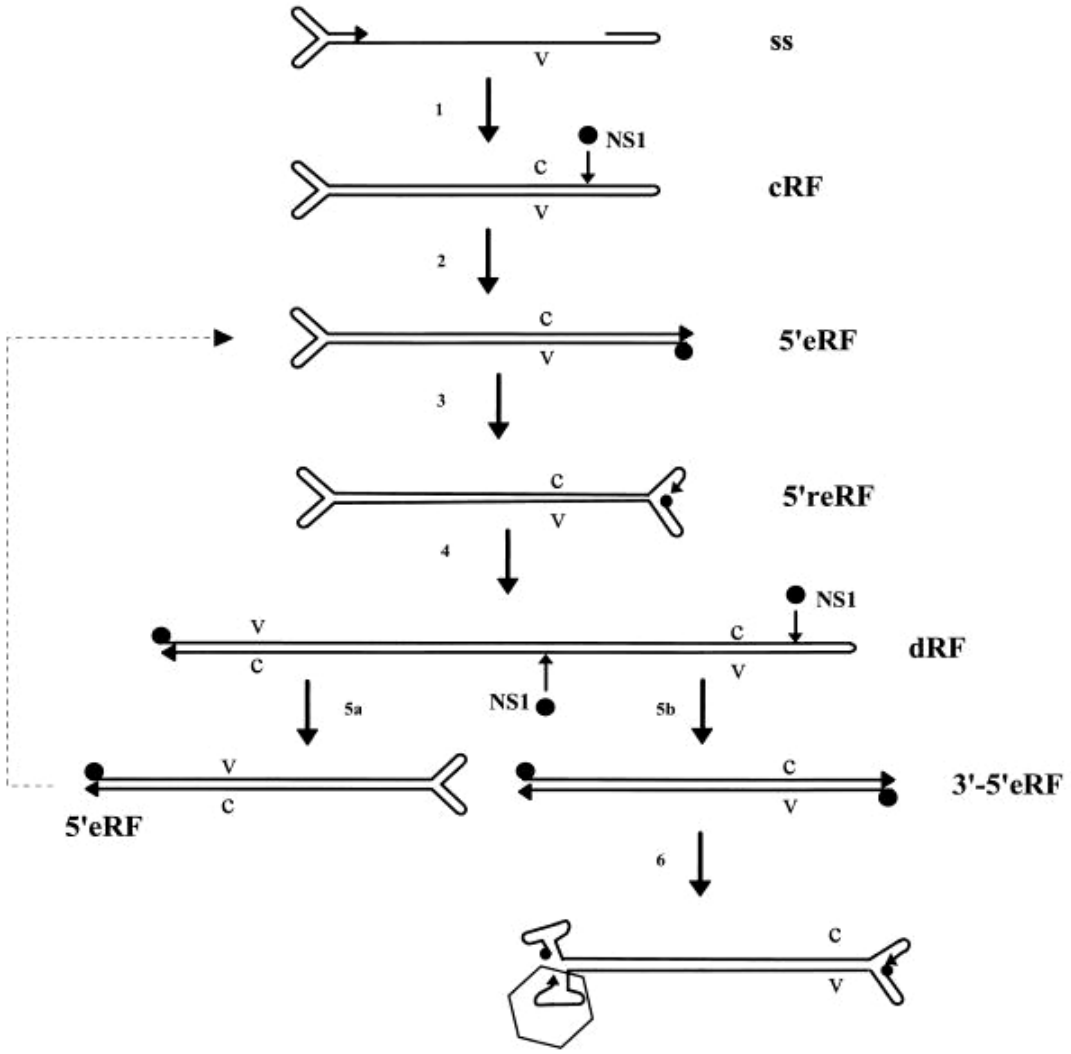


Figure 6: Modified rolling hairpin model for MVM DNA replication. NS1 is depicted as a small filled circle. Small arrowheads indicate DNA 3′ ends. ss: ssDNA; eRF: extended monomeric RF; v: viral strand; c: complementary strand. The open polygon in step 6 represents the capsid (reprinted from Kuntz-Simon, *et al.,* 1999).

As mentioned before, the dimer junction is resolved by a mechanism termed “junction resolution”. As shown in Figure 7, initially, the left-end sequence of MVM folds into a Y-shaped hairpin and a duplex stem region interrupted by a mismatched “bubble” sequence. This bubble is a potential origin sequence, but it cannot be nicked by NS1 in this hairpin configuration. The rolling-hairpin displacement synthesis proceeds, and the hairpin is unfolded and copied to create the fully base-paired palindromic junction, spanning adjacent genomes in dimer RF. Now, the dinucleotide bubble sequence, serves as an active origin of replication, oriLTC, while the analogous sequence containing the bubble trinucleotide, oriLGAA, is completely inactive (Cotmore and Tattersall, 1998).

The NS1 protein can bind by itself, but it cannot nick the origin, it requires the cooperation of a cellular factor called PIF, Parvovirus Initiation Factor. PIF binds to a repeated ACGT motif located immediately next to the NS1-binding site and activates the NS1 endonuclease function in some as yet undefined way (Cotmore and Tattersall, 1998; Christensen and Tattersall, 2002).

Individually, PIF and NS1 bind equally well to both forms of oriL; however, in combination, they show synergistic binding to oriLTC, but not to oriLGAA, leading to asymmetric initiation of DNA replication across the dimer junction. The minimal active origin is a 50-bp sequence which contains three distinct recognition elements: an NS1 nick site (CTWWTCA); an NS1 binding site (ACCA)2 sequence, and a third block of sequence which contains a consensus ATF (activated transcription factor) binding site (Burnett and Tattersall, 2003).

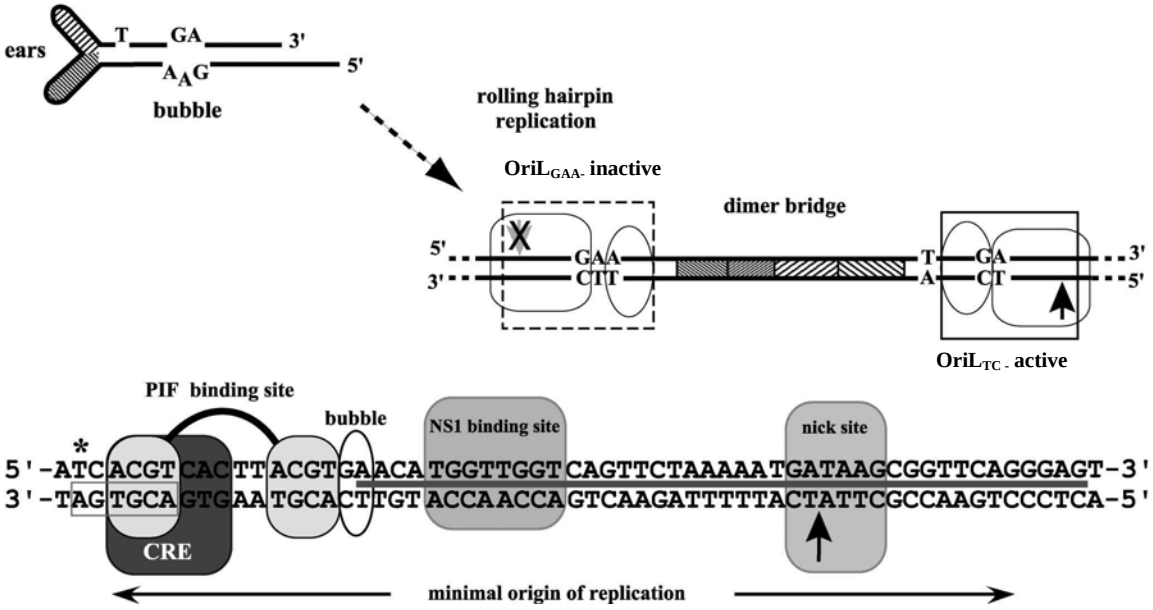


Figure 7: The dimer junction resolution and minimal origin of replication. The left-hand hairpin of MVM is shown in diagrammatic form. The small ears and bubble sequence are indicated. The dimer junction generated by rolling hairpin replication, is shown on the right. The palindromic sequences derived from the hairpin ears are represented by cross-hatched boxes. The minimal active origin is boxed, with an arrow indicating the nick site. 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 (reprinted from Burnett and Tattersall, 2003).

- **LuIII Replication**

Unlike MVM, the model of replication of LuIII lacks the arrangement of the domains present in the intermediate form MVM which has only active origin form in the left hairpin (Cotmore and Tattersall, 1994). Also, MVM needs the dimer junction to replicate and synthesize the minus or plus strand (Cotmore and Tattersall, 1998).

Diffoot *et al*. (2005) proposed that the left hairpin has a function in replication, suggesting that this LuIII 3' sequence, contains all the cis-acting sequence required for excision and DNA replication. The results suggest that the left hairpin of LuIII has an active NS1 driven origin of replication and it is proposed that the plus and minus DNA strands independently initiate replication from the right and left hairpins respectively and result in an equivalent amount of both viral DNA strands. The possible mechanism for the replication of LuIII is shown in Figure 8. The model proposes that the plus strand starts replication from the right hairpin and the minus strand from the left hairpin (step 1) suggesting that both hairpins can serve as primers, which 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). NS1 opens the cRF by nicking the viral strand (step 2). There are active NSI nick sites present at both termini of parvovirus LuIII that differ from each other (Diffoot *et al*., 1993). Those nicks generate two origins of replication running in opposite directions that lead to strand displacement. Following the NS1 nick, there is a displacement and copying of the strand (step 3). That promotes the development of an extended molecule containing covalently joint viral and complementary DNA strands (step 4).

An earlier study (Diffoot *et al.*, 1989) 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 suggests that in each strand there exists both flip and flop conformations at both termini (step 5) (Diffoot *et al*., 2005).

A. Replication of LuIII using plus strand

B. Replication of LuIII using minus strand

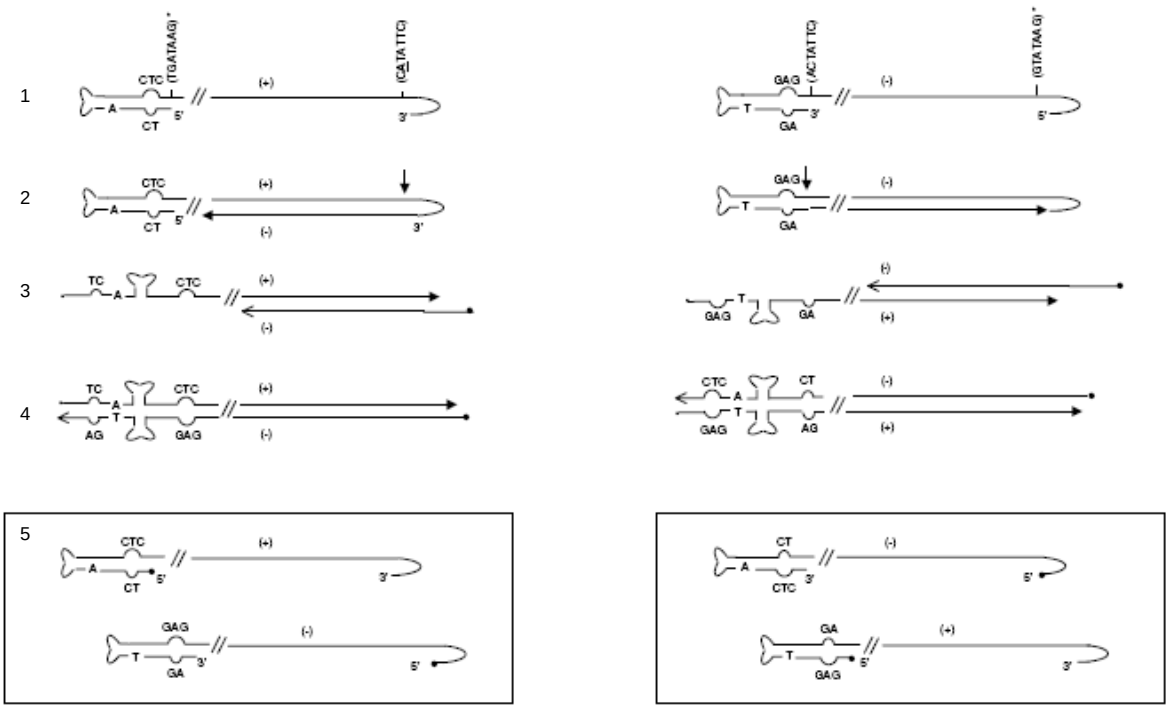


Figure 8: Proposed Model for the Replication of Parvovirus LuIII. **A:** Replication of the (+) strand, **B:** replication of the (-) strand.The NS1 nick site and its complementary sequence are indicated (*).The bubble sequence at the left hairpin is indicated. The arrows point to the NS1 nick sites. The flip and flop conformation from both strands are boxed. (Diffoot *et al*., 2005)

As it was mentioned, when comparing the LuIII and MVM sequences, an AT rich sequence is present only in LuIII, which may have function in the encapsidation process (Diffoot *et al.,* 1993; Corsini *et al.,* 1995) and may also have a function as a replication origin in yeast. Previous studies support this hypothesis (Román-Pérez, 2000; Arroyo, 2000; De Jesús, 2004).

LuIII A/T rich sequence

The budding yeast *Saccharomyces cerevisiae* has the best characterized replication origins among eukaryote models. The origin of replication, also called the replication origin, is a particular DNA sequence where DNA replication is initiated and it may proceed from this point bidirectionally or unidirectionally. Autonomously replicating sequences (ARS) are defined operationally as sequences that support the maintenance of a plasmid in growing yeast cells (Breier *et al*., 2004). It has been demonstrated that sequences consisting of more than three consecutive adenine or thymine without guanine or cytosine are crucial for ARS function (Yukiko Okuno *et al*., 1999). The specific structure of the origin of replication varies somewhat from species to species, but all share some common characteristics. The ARS consist of a 100 to 200 bp sequence with a high AT content (73 to 82%) (Newlon, 1988). The core consensus of ARS is 5'-[A/T]TTTAT[A/G]TTT[A/T]-3' and additional sequences 3' to the T-rich strand of the consensus. The origin of replication binds the pre-replication complex, a protein complex that recognizes, unwinds, and begins to copy DNA (Palzkill and Newlon, 1988).

At the core of every yeast replication origin is a replicator sequence containing a conserved 17 bp stretch know as the ARS consensus sequence, ACS. The ACS is required for binding of the initiator protein, the origin recognition complex, ORC. ORC is a multifunctional heterohexameric ATPase conserved among eukaryotes (Breier *et al*., 2004).

The ARS sequence is composed of two to three domains. The most important is the central ACS or **domain A** (Figure 9). The other is the **domain B**, a region upstream

of the AT rich of the ACS. This domain exhibits little sequence conservation among ARSs and is composed of several nonredundant sequence elements (B1, B2, B3, B4), which vary from one ARS to another (Theis and Newlon, 1997). Some, but not all, mutations in the B1 sub-element can reduce the efficiency of ORC binding, suggesting that B1 functions with domain A as part of the ORC binding site. The B2 sub-element, or DUE (DNA Unwinding Element), is AT-rich and serves as the site for the initial DNA unwinding, allowing the replication machinery to enter the DNA duplex in this area and assemble the first new nucleotides. The B3 sub-element is a 12-bp degenerate sequence that acts as a binding site for the transcriptional activator/repressor ARS binding factor 1 (ABF1). The function of the B4 sub-element is as yet unknown. Some ARSs also have stimulatory sequences upstream of domain A (on the opposite side of the ACS) known as domain C, as well as binding sites for the Rap1p transcription factor (Theis *et al.*, 1997).

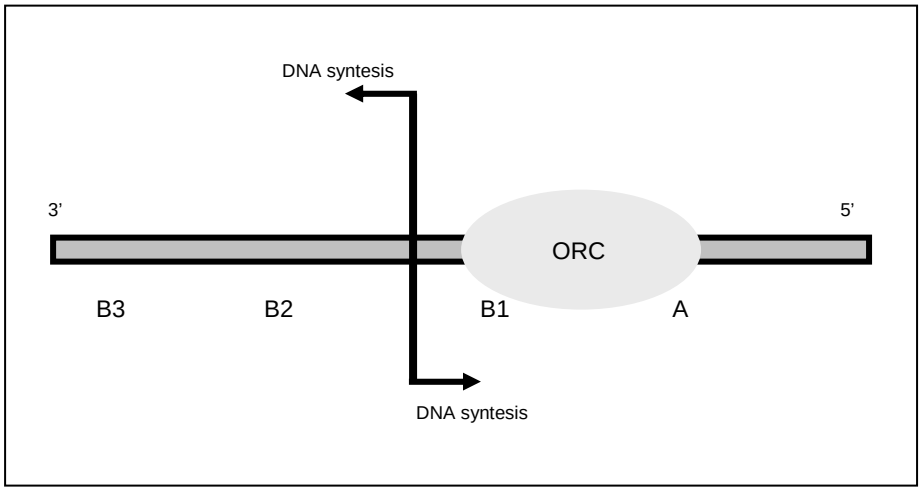


Figure 9: Modular structure of ARS1 in *Sacharomyces cerevisia*e. ARS 1 In yeast has a modular structure and is composed of A and B domains. A and B1 comprise the ORC binding site, B2 is a DNA-unwinding element, and B3 is the target site of the transcription factor ARS-binding factor 1 (reprinted from De Jesús thesis, 2004).

Given the homology observed from the A/T rich sequence of LuIII with ARS sequences in yeast it has been proposed that this A/T sequence could represent an origin of replication at the right terminus of the viral genome required for the synthesis of the plus strand viral DNA.

An origin of replication has been identified at the 3' terminus of the plus strand of LuIII (Diffoot *et al*., 1989; Diffoot *et al*., 1993). This origin of replication consists of the 3' hairpin and is responsible for the synthesis of the minus strand of LuIII. Given the encapsidation pattern of LuIII (50% minus strand, 50% plus strand) an origin of replication must be present at the right end of the minus strand. Replication from this origin would result in the synthesis of the plus strand (Diffoot *et al*., 1989, 1993, 2005).

The analysis done by Arroyo (2000) indicated that only the 47 bp of LuIII AT rich sequence can act as a replication origin but in a weakly form, suggesting that the right terminal could contain other elements necessary for the proper function of this sequence like an ARS. De Jesús (2004) studied the sequence comprised between the map unit 88 to 100 of LuIII genome and revealed that this region can function like an autonomous replication sequence in yeast. With the purpose to continue the work of De Jesús and knowing that the virus infects eukaryotic cells in continuing dividing stage (S phase), we test the possible role of this region allowing replication in HeLa cells. Thus, a plasmid containing the sequence present at the right end (map units 88-100) of the parvovirus LuIII genome was constructed and its replication in higher eukaryotic cells was studied.

Objectives

The objective of this research is to determine if the AT- rich region and other sequences in map units 88-100 present in the right end of the parvovirus LuIII genome can function as an origin of replication in higher eukaryotic cells. In order to accomplish this objective it is necessary to:

- Construct a genomic clone pDsRed LuIII 88-100 that contains the DsRed sequence that codes for the red protein, the CMV promoter upstream the DsRed, sequence, the resistance to ampicillin gene, a prokaryotic replication origin, and the parvovirus LuIII AT-rich region from map unit 88 to 100.

- Transfect the plasmid pDsRed LuIII 88-100 into HeLa cells.

- Determine if the LuIII A/T rich sequence can function as an ARS through HeLa cells transfection, counting the cells in a flow cytometer, and molecular analysis.

CHAPTER III

Materials and Methods

Plasmids Construction

The aim of this research is to determine if the A/T rich region in the LuIII viral genome is capable of acting as a replication origin in higher eukaryotes. To do so, three plasmids were constructed: pDsRed CMV or positive control, pDsRed CMV (-SV40) or negative control and pDsRed LuIII 88-100. The construction of an intermediary plasmid pd2EGFP LuIII 88-100, was also necesary.

- Construction of pDsRed CMV

The Cauliflower Mosaic Virus promoter (CMV), was extracted from the pd2GFP-1 CMV plasmid (Figure 10 A), a preexisting plasmid in the laboratory, and incorporated into the pDsRed2-1(Figure 10 B) (BD Biosciences Clontech, Palo Alto, CA). The preexisting plasmid originally was pd2GFP-1 (BD Biosciences Clontech, Palo Alto, CA) in which the CMV promoter was incorporated in the *Sal* I- *Bam* HI multiple cloning site. The pDsRed2-1 plasmid encodes the red fluorescent protein called DsRed (Dicosoma species “Red”).

The plasmid pDsRed2-1, and the pd2EGFP-1 CMV were digested with the restriction enzymes *Bam* HI (G/GATCC) and *Eco* RI *(*G/AATTC) from Roche Molecular Biochemical, Indianapolis.

The digested pd2EGFP-1 CMV and pDsRed2-1 were electrophoresed on a 1% agarose gel (Fisher, Molecular Bioloy Grade Agarose, BP1356-100) in 1X TAE (40mM Tris-acetate, 2mM EDTA, pH 8.5) buffer at 70V (Figure 11). Gel slices containing the fragments of 986 bp from the pd2EGFP-1 CMV digestion and the 4076 bp fragment from the pDsRed2-1digestion were extracted and purified from the gel using the Gene Clean Spin Kit® (QBio-gene, 1101-200, Montréal, Canada). Extracted fragments were

ligated using T4 DNA ligase (Roche Molecular Biochemicals, 11635379001, Indianapolis).

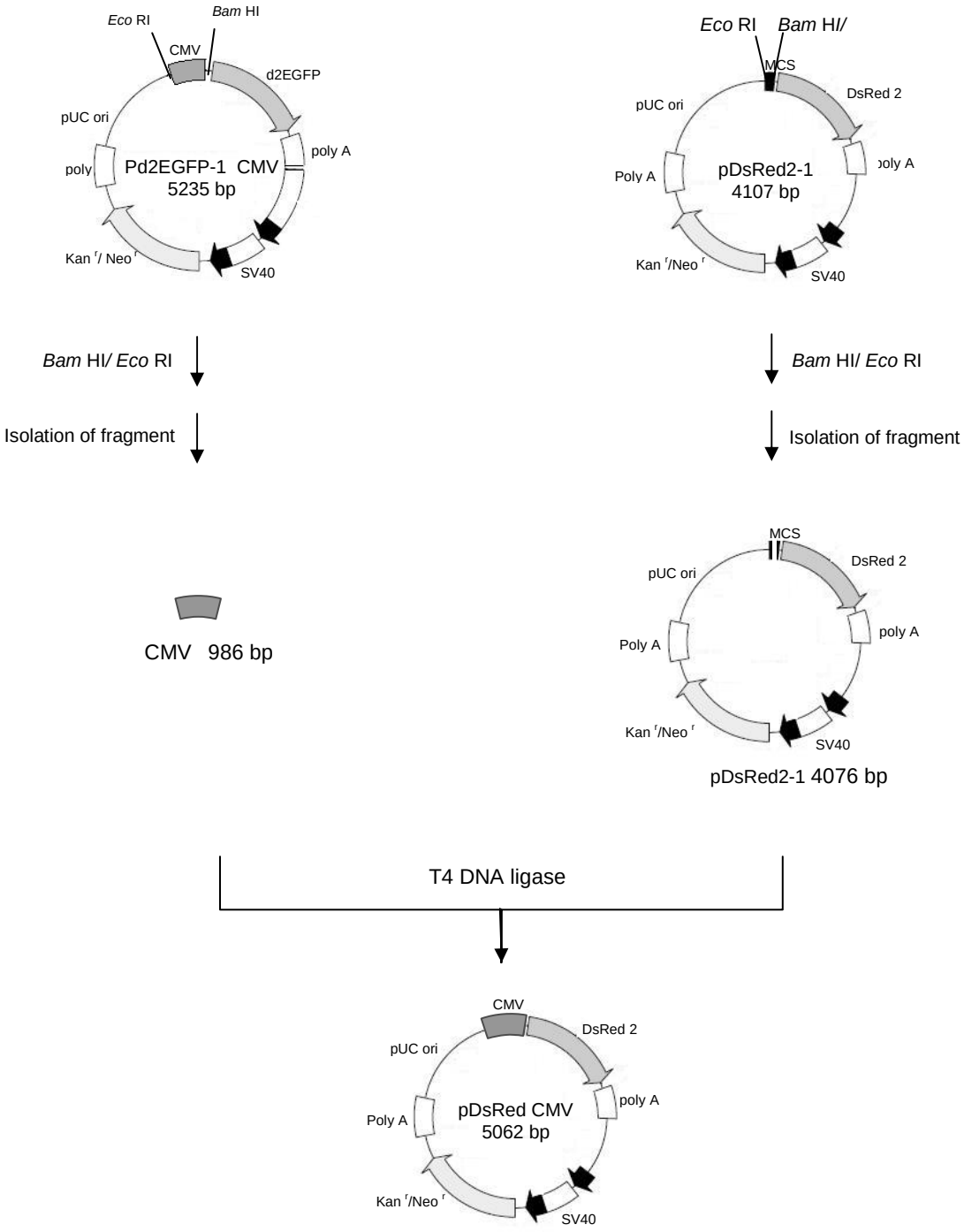


Figure 10: Strategy used to construct of pDsRed CMV (positive control). Restriction sites used, the fragment isolated to be ligated and the different genes present in the plasmid are shown. The CMV promoter are cloned into the *Bam* HI and *Eco RI* sites of pDsRed2-1.

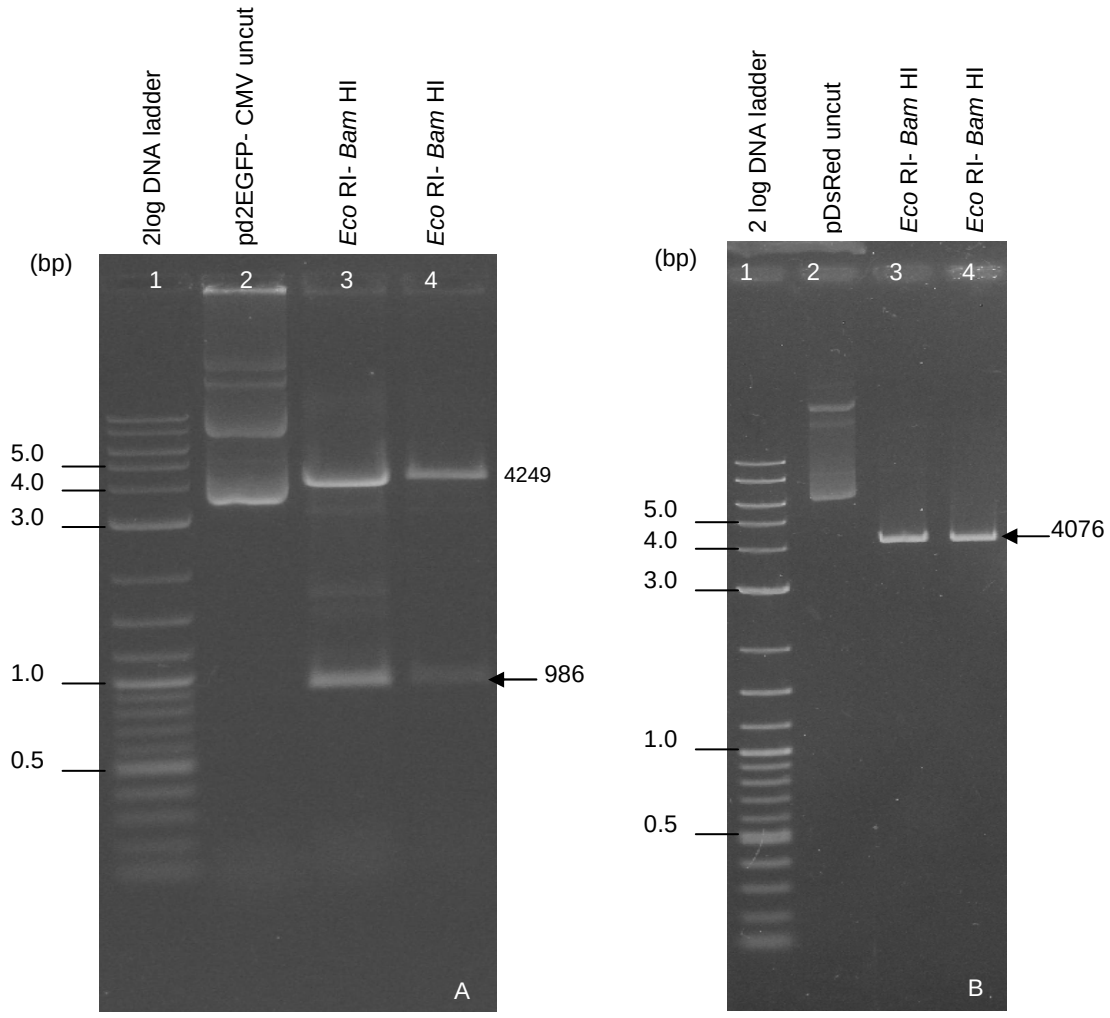
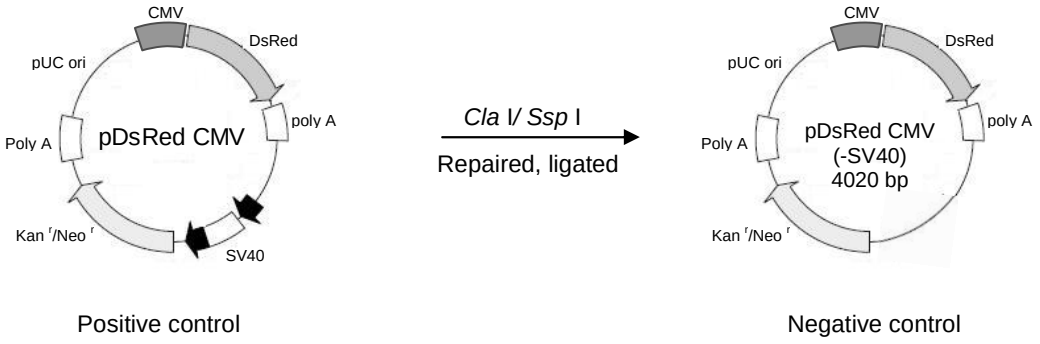


Figure 11: Digestion of pd2EGFP-1 CMV and pDsRed2-1 with the restriction enzymes *Eco* RI and *Bam* HI. **A:** Digestion of pd2EGFP-1 CMV. **B:** Digestion of pDsRed2-1. Samples were electrophoresed on 1% agarose gel in TAE 1 X buffer at 70V. Desired fragments, 986bp and 4076bp are indicated with arrows.

- Construction of pDsRed CMV(-SV40)

The pDsRed CMV(-SV40), negative control, was constructed digesting the positive control with *Cla* I (AT/CGAT) and *Ssp* I *(*AAT/ATT) to eliminate the replication origin, SV40 (Figure 12). The digestion generated three fragments, 4130 bp, 553 bp, and 379 bp. The digested plasmid was electrophoresed on a 1% agarose gel in 1X TAE buffer at 70V (Figure 13). Gel slice containing the fragment of 4130 bp was extracted and purified by the Gene Clean Spin Kit®. This fragment had to be repaired with Klenow enzyme (Roche, Nutley, NJ), and then ligated using the T4 DNA ligase.



Positive control

Negative control

Figure 12: Strategy used to construct of pDsRed2-1 CMV (-SV40) (negative control). Restriction sites used and the different genes present in the plasmids are shown. The SV40 replication origin was eliminate.

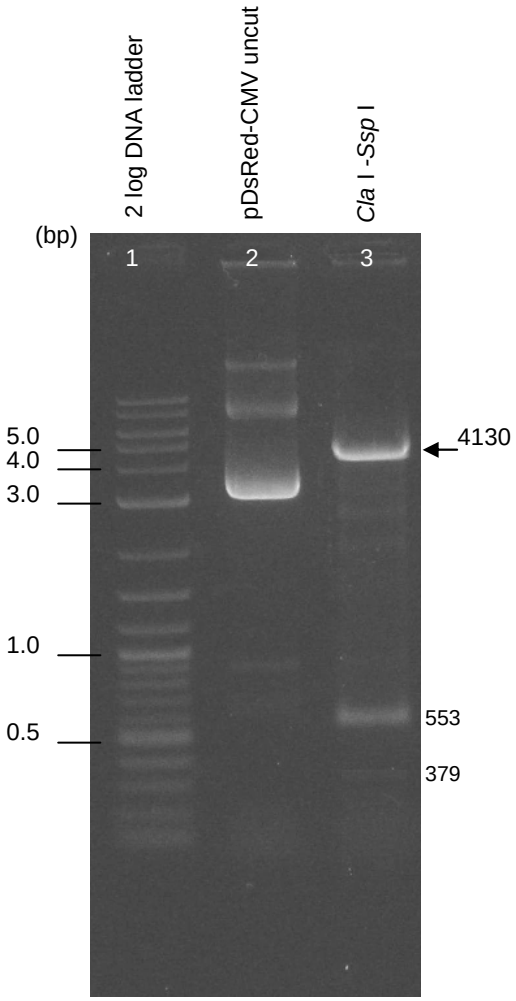


Figure 13: Digestion of pDsRed-CMV with *Cla* I- *Ssp* I. Samples were electrophoresed on 1% agarose gel in TAE 1 X buffer at 70V. The desired fragment of 4130 bp is indicated with an arrow.

- Construction of pd2EGFP LuIII 88-100

In order to construct the plasmid pDsRed-LuIII 88-100 an intermediary plasmid pd2EGFP LuIII 88-100 was necessary. The plasmid pGLu883ΔXba was treated with the enzymes *Xba* I *(*T/CTAGA)*,* and *Eco* RI, (Roche, Nutley, NJ) to obtain the A/T rich region, at m.u. 88-100 of LuIII (Figure 14). As the same way, the pd2GFP plasmid was also digested with *Xba* I and *Eco* RI (Figure 14 B).

The digested pd2EGFP and pGLu883ΔXba plasmid were electrophoresed on a 1% agarose gel (Fisher, Molecular Bioloy Grade Agarose, BP1356-100) in 1X TAE (40mM Tris-acetate, 2mM EDTA, pH 8.5) buffer at 70V (Figure 15). Gel slices containing the fragments of 2588 bp from pd2EGFP and the 663 bp fragment from pGLu883ΔXba were extracted and purified from the gel using the Gene Clean Spin Kit®. Extracted fragments were ligated using the T4 DNA ligase.

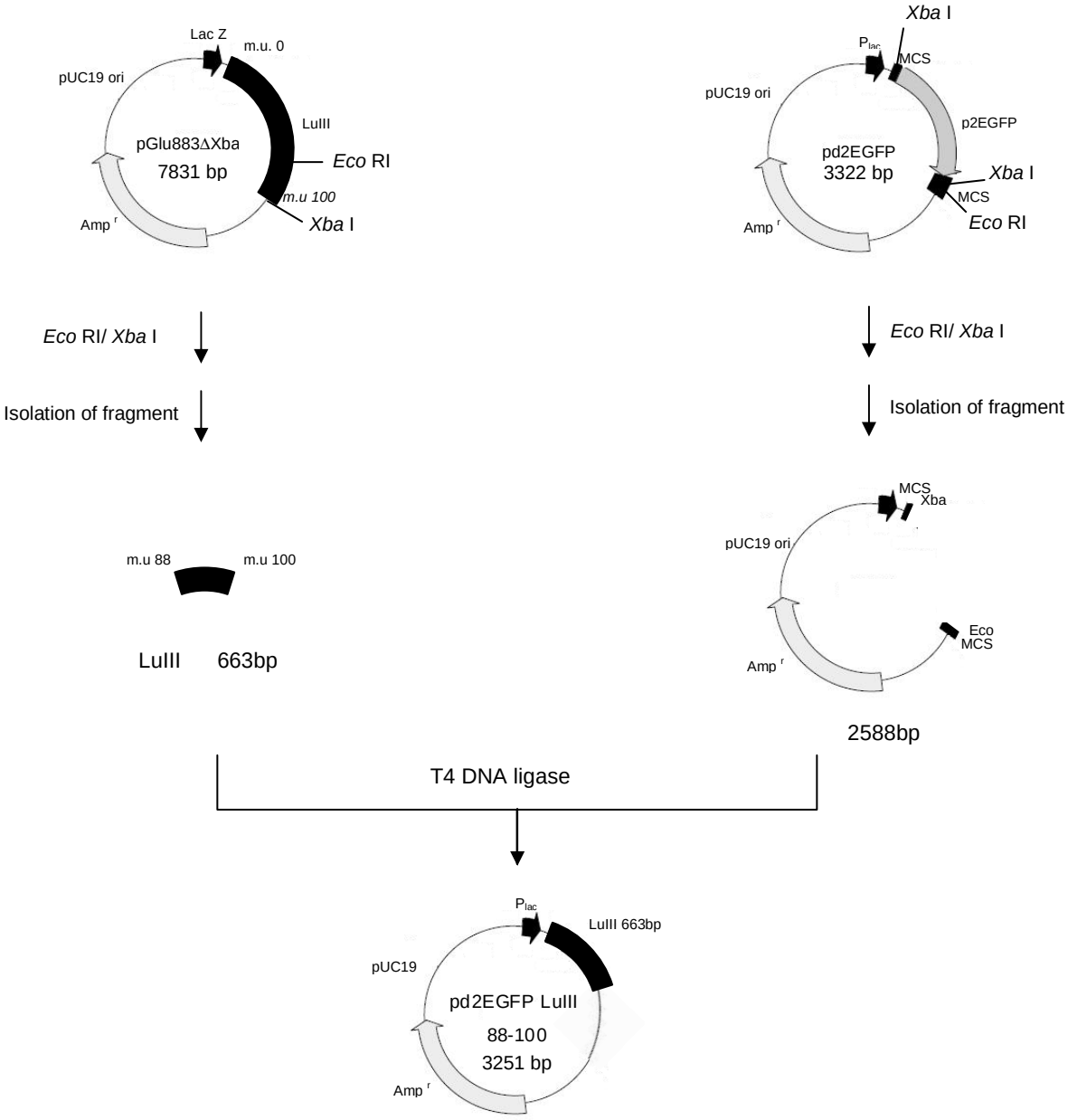


Figure 14: Strategy used to construct of pd2EGFP LuIII 88-100. Restriction sites used, the fragment isolated to be ligated and the different genes present in the plasmid are shown. The m.u 88-100 of LuIII is cloned into the *Eco* RI and *Xba* I sites of pd2EGFP plasmid.

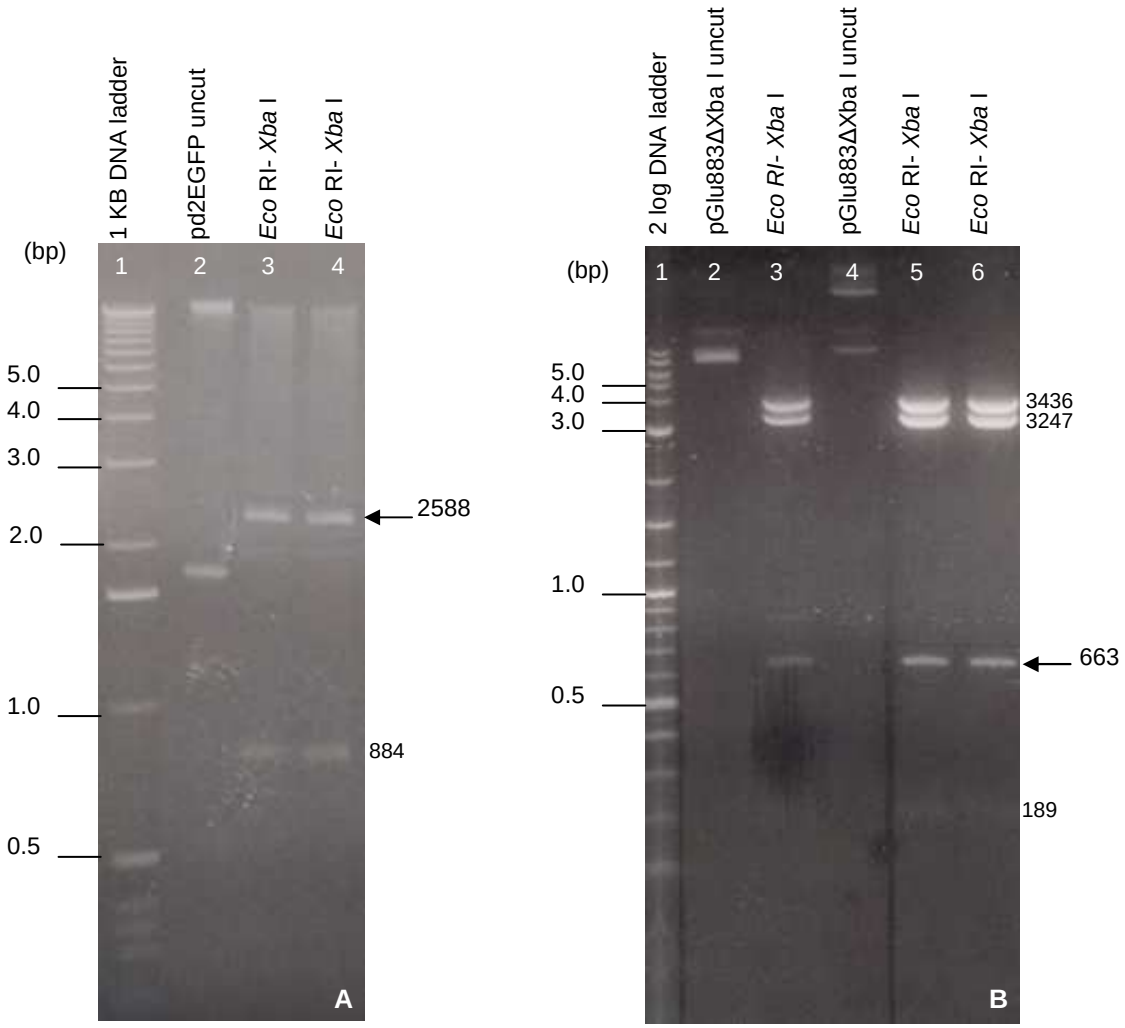


Figure 15: Digestion of pd2EGFP and pGLu883ΔXba with *Eco* RI- *Xba* I. **A**: Digestion of pd2EGFP. **B**: Digestion of and pGLu883ΔXba. Samples were electrophoresed on 1% agarose gel in TAE 1 X buffer at 70V. The desired fragments are indicated with arrows.

- Construction of pDsRed LuIII 88-100

To construct the pDsRed LuIII 88-100 plasmid, the plasmid pd2EGFP LuIII 88-100 was digested with *Eco* RI (Roche, Nutley, NJ) and *Stu* I *(*AGG/CCT) (Fermentas*,* Hanover, MD) (Figure 16 A) generating the fragments of 3161 and 60 bp. The pDsRed CMV plasmid was digested with *Eco* RI, *Ssp* I (AAT/ATT) (Roche, Nutley, NJ) (Figure 16 B), generating the fragments of 2863, 1606, and 593 bp.

The digested pd2EGFP LuIII 88-100 and pDsRed CMV were electrophoresed on a 1% agarose gel in 1X TAE buffer at 70V (Figure 17). Gel slices containing the desired fragments were extracted and purified from the gel using the Gene Clean Spin Kit®. The fragments were ligated using the T4 DNA ligase.

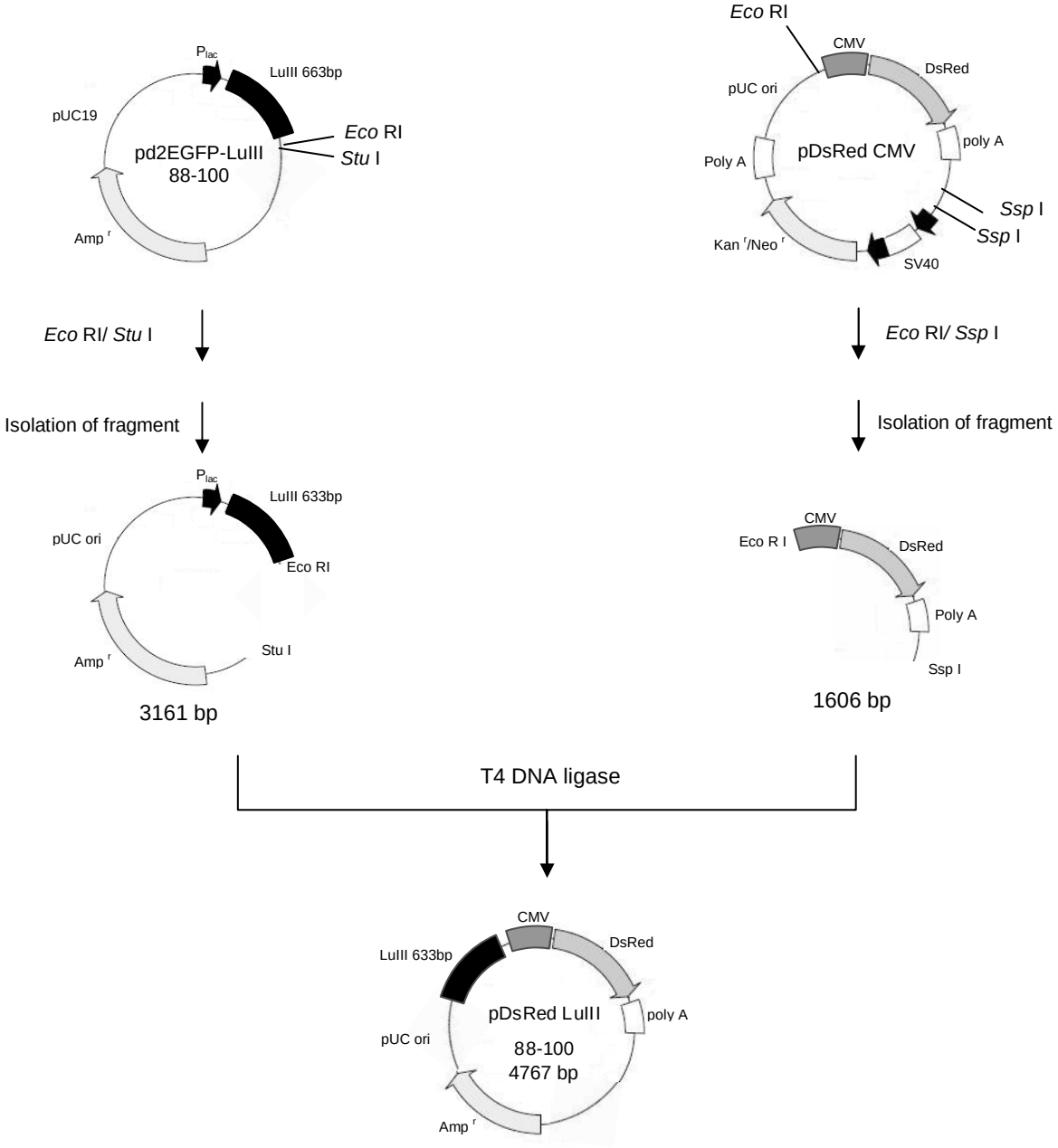


Figure 16: Strategy used to construct of pDsRed LuIII 88-100. Restriction sites used, the fragment isolated to be ligated and the different genes present in the plasmid are shown. The fragment of 3161 bp is cloned into the *Eco* RI and *Ssp* I sites of pDsRed CMV plasmid.

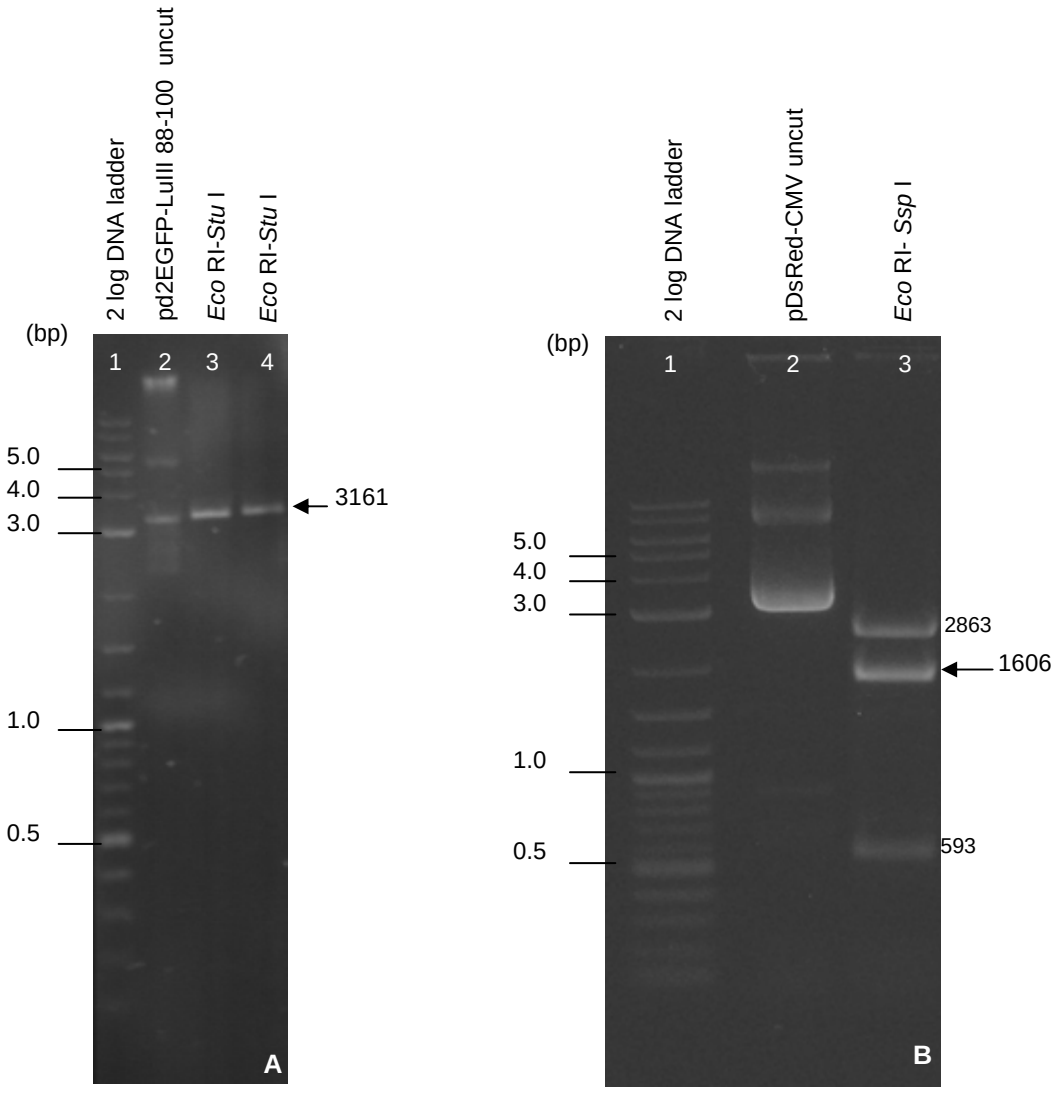


Figure 17: Digestion of pd2EGFP LuIII 88-100 and pDsRed CMV. **A**: Digestion of pd2EGFP LuIII 88-100 with *Eco* RI and *Stu* I. **B**: Digestion of DsRed CMV with *Eco* RI and *Ssp* I. Samples were electrophoresed on 1% agarose gel in TAE 1 X buffer at 70V. The desired fragments are indicated with arrows.

Staggered fragments were ligated overnight at 15ºC using 1U of T4 DNA Ligase (Roche Molecular Biochemicals, 11635379001, Indianapolis). The fragments with blunt ends were ligated at 25ºC with 4U of T4 DNA ligase overnight. The resulting molecules were used to transform competent cells by the heat-shock protocol (Ausubel *et al.*, 2005), and the resulting clones were characterized by restriction digestion.

Preparation of Competent cells

Competent cells were prepared inoculating a single colony of *E. coli DH5α* (φ80dlacZΔM15, recA1, gyrA96, thi-1, hsdR17 (rk -, mk+), supE44, relA1, deoR, Δ(lacZYA-argF)U169) into 2 ml of LB (1% Bacto® tryptone, 0.5 % Bacto® yeast extract, 86 mM NaCl and 1 M NaOH) broth. Cells were grown overnight at 37ºC with constant shaking. An aliquot of 1 ml was transferred into 100 ml of LB media and subsequently incubated at 37ºC with constant shaking between three and four hours. Cells were centrifuged at 1080 x g for 10 minutes at 4ºC. The supernatant was discarded; the pellet was gently resuspended in 25 ml of cold 50 mM $CaCl_2$ and placed in ice for 30 minutes. Cells were then centrifuged at 1080 x g for 10 minutes and the pellet recovered was resuspended in 5 ml of cold 50 mM $CaCl_2$/ 20% glycerol, and aliquots of 100 µl were prepared into sterile microtubes.

Transformation of Competent cells

The transformation of *E. coli DH5α* competent cells was done with approximately twenty-five nanograms of the respective ligation mixture. Cells with no DNA were used as a negative control, and teen nanograms of pUC 19 were used for a transformation control (Table 3).

After adding the DNA to the competent cells, the tubes were placed on ice for 30 minutes and subsequently heat-shocked for 2 minutes at 42ºC. Cells were then incubated on ice for 2 minutes. Afterward, 100 µl LB medium without antibiotics was added to the samples and incubated for one hour at 37ºC with shaking at 225 rpm. *DH5α* transformed cells were spread on LB agar plates containing the corresponding antibiotic (ampicillin: 50 µg/ml, kanamycin: 30 µg/ml) and then incubated overnight at 37ºC.

Table 3: **Transformation of *E. coli DH5* 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 Antibiotic control	None	0	LB kanamycin
4 Transformation control	pUC19	10	LB ampicillin
5 -7 Experimental	pDsRed CMV (positive control)	20	LB kanamycin
		30	
		50	
8 -10 Experimental	pDsRed CMV (-SV40) (negative control)	20	LB kanamycin
		30	
		50	
11-13 Experimental	pDsRedLuIII 88-100	20	LB ampicillin
		30	
		50	

Plasmid DNA Extraction and Restriction Characterization.

Transformants were inoculated in 5 ml of LB broth with kanamycin or ampicillin, and incubated overnight at 37ºC with constant shaking. The plasmid DNA was extracted and purified from the culture using the alkaline lysis minipreparations purification

protocol (Small Scale Plasmid Purification Protocol) (Ausubel *et al*., 2005). Once the restriction characterizations were done, one molecule of each plasmid was selected to be used in the transfection assay.

Tissue Culture

Transfection experiments were performed in HeLa cells. HeLa (ATCC, Rockville, MD) cells were grown in 90% Minimal Essential Medium 1X (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 culture medium was buffered to a neutral pH of 7 with 0.75N HCl and 7.5% $NaHCO_3$. They were incubated at 37ºC in 25 cm^2 plastic tissue culture flasks.

For sub-culturing, the cells were rinsed twice with phosphate buffered saline 1X (PBS) and incubated in 1X Trypsin (Difco, Detroit, MI) for 5 min at 37ºC. Cells were harvested by centrifugation at 2490 x g for 5 min at 4ºC. The resultant pellet was resuspended in the medium described above and seeded into 25 cm^2 culture flasks at a rate of 1:3.

Transfection Assay

HeLa cells were transfected by the electroporation method (Maxwell and Maxwell, 1988). The HeLa cells were maintained in a 25 cm^2 plastic culture flask in culture 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.

Cells were washed three times with PBS 1X and then tripsinized at 37ºC for 5 min. Cells were harvested by centrifugation at 2490 x g for 5 min at 4ºC. The pellet was resuspended in 10 ml PBS and centrifuged as described above.

The pellet was resuspended in 7.2 ml 1X MEM and a concentration approximately of 10^7 cells/ ml (800 µl) was 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 and placed in the incubator at 37ºC. The flasks were maintained in the dark at all times. Medium was replaced 24 hrs after transfection, and the cells were incubated at 37ºC and observed at 24, 48 and 72 hours.

All experiments were performed in duplicate.

Fluorescent Microscopy

Samples were observed at 24, 48 and 72 hr 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

Samples were countered 72 hrs post-transfection using a BD FACSAria Flow Cytometer (BD Biosciences).

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, and the cells were counted. The measurement was done in duplicate.

DNA Extraction from HeLa cells

After 6 days of transfection the cells were passaged, and 4-6 days later, the DNA was isolated by the Hirt extraction procedure. This method is employed to extract selectively low-molecular weight DNA, as described by Tam and Astell (1994). Cells were rinsed with 1X PBS buffer before lysis with transfection solution for 15 min in constant shaking. The viscous lysate was transferred to 1.5 ml microtubes and 140 µl of 5N NaCl was added. After overnight incubation on ice, samples were centrifuged 12,000 x g for 20 min. Supernatants were digested with 0.5 mg of proteinase K per ml overnight at 50ºC before extraction with phenol/ chloroform/ isoamyl alcohol, and chloroform-/isoamyl alcohol, and precipitated with 2 volumes of isopropanol. DNA was resuspended in 30 µL TE (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) and treated with RNase (1µg/µl) for 1 hour at 37ºC.

The extracted DNA was treated with the restriction enzymes *Dpn* I (GmA/TC) (New England Biolabs, Beverly, MA) and *Mbo* I (/GATC) (New England Biolabs, Beverly, MA). These enzymes recognize the same sequence of DNA, but *Dpn* I only cuts the DNA if it is methyled, whereas *Mbo* I cuts if the DNA is not methyled.

CHAPTER VI

Results and Discussion

The DNA sequence of LuIII reveals the presence of an adenine-thymine (A/T) rich region at map unit 89 (5'-TATGCTCTATGCTTCATATATATTATAATATTATTATAC TAACTAA-3'), which is unique to the viral genome and has not been found in any other parvovirus sequenced to date. This A/T rich sequence shares a similarity with the autonomously replicating sequences (ARS) described for *Sacharomyces cerevisiae* (Arroyo, 2000). These ARS consist of an A/T rich region containing discrete sites in which mutations have been shown to affect origin function. Transformation of a plasmid containing just the 47 bp of the LuIII A/T sequence into *S. cerevisiae* demonstrated that the LuIII A/T rich sequence could direct autonomous replication in a weakly manner (Arroyo, 2000). Given these results De Jesús constructed a plasmid that contained this 47 bp sequence and the right terminal of LuIII genome (map unit 88-100). Replication of this new plasmid demonstrated that this sequence of LuIII (m.u 88-100) could act as a replication origin in yeast (De Jesús, 2004).

In order to investigate if LuIII can acts as an origin of replication in higher eukaryotes the following plasmids were constructed:

- **pDsRed CMV**: reporter gene DsRed2; SV40 replication origin for eukaryotes; puC 19 replication origin for prokaryotes; Neomycin/ Kanamycin resistance.
- **pDsRed CMV (-SV40)**: reporter gene DsRed2; puC 19 replication origin for prokaryotes; Neomycin/ Kanamycin resistance.
- **pDsRed LuIII 88-100**: reporter gene DsRed2; puC 19 replication origin for prokaryotes; LuIII 88-100 sequence; Ampicilin resistance.

Transformation of the recombinant clones

The bacterial transformation was done in three independent assays with the corresponding DNA samples. In all transformations the control plates (cells without

DNA), resulted in normal growth when plated without antibiotics, demonstrating that the cells were viable after the heat shock. Cells without DNA resulted in no growth, demonstrating that the cells were sensitive to the antibiotics and were not contaminated. All transformation assays had a low efficiency in comparison to the expected 10^7 CFU/ µg DNA for the $CaCl_2$ method. This may be due to the quality of the DNA or the complexity of the plasmids.

- pDsRed-CMV clone

Transformation of *E.coli* DH5α cells with the possible pDsRed CMV plasmid resulted in a total of 56 colonies, ten colonies were analyzed and all of them were positive for the expected construction. The transformation efficiency was 2.1 $x10^5$ CFU/ µg of DNA.

- pDsRed CMV(-SV40) clone

Possible recombinant molecules of pDsRed CMV (-SV40) were transformed into *E.coli DH5α* competent cells with an efficiency, of 2 $x10^5$ CFU/µg. Ten individual recombinant colonies were obtained. Five of ten analyzed colonies corresponded to the desired plasmid.

- pDsRed LuIII 88-100 clone

Transformation of *E. coli DH5α* cells with the pDsRed LuIII 88-100 recombinant molecules resulted in a transformation efficiency of 2.4 x 10^5 CFU/µg of DNA. Eight colonies were obtained from the transformation of which five corresponded to the desired plasmid.

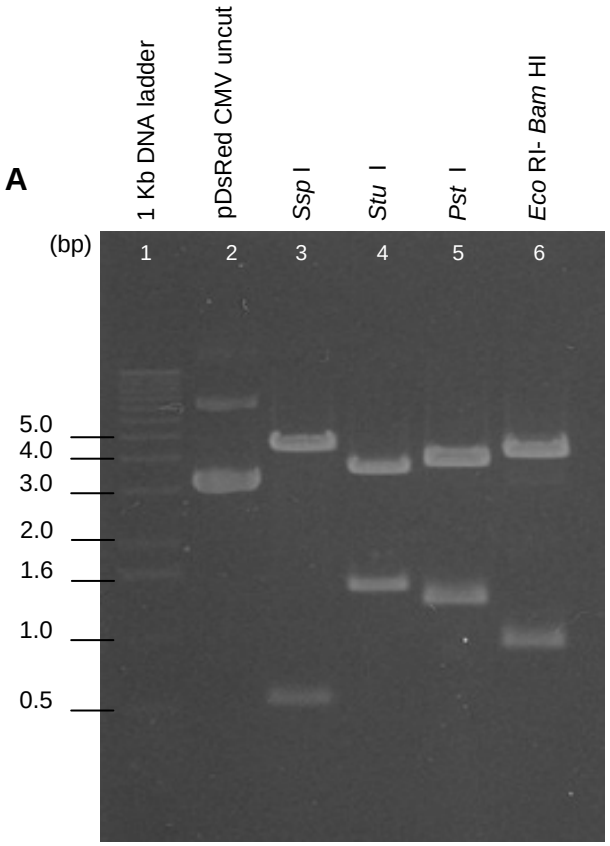
Restriction Analysis of recombinant clones

DNA samples were digested with the corresponding restriction enzymes and analyzed by gel electrophoresis, as described in the Material and Methods chapter (page 49).

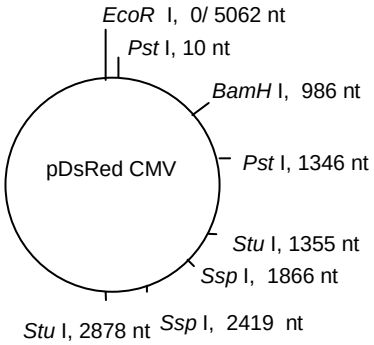
- pDsRed CMV recombinant clone

Digestions of the positive control with the *Ssp* I which has two recognition sites in the SV40 sequence, generated two fragments of 4509 bp and 553 bp as shown in Figure 18A lane 3. *Stu* I digest at the end of the DsRed sequence and at the end of SV40, generating two fragments of 3539 bp and 1523 bp as shown in lane 4. *Pst* I digest two times, one at the beginning of CMV and the second in the DsRed sequence, generating two fragments as observed in lane 5; the *Eco*R I-*Bam*H I restriction analysis generated the 986 bp and 4076 bp fragments, as shown in lane 6.

A



B



C

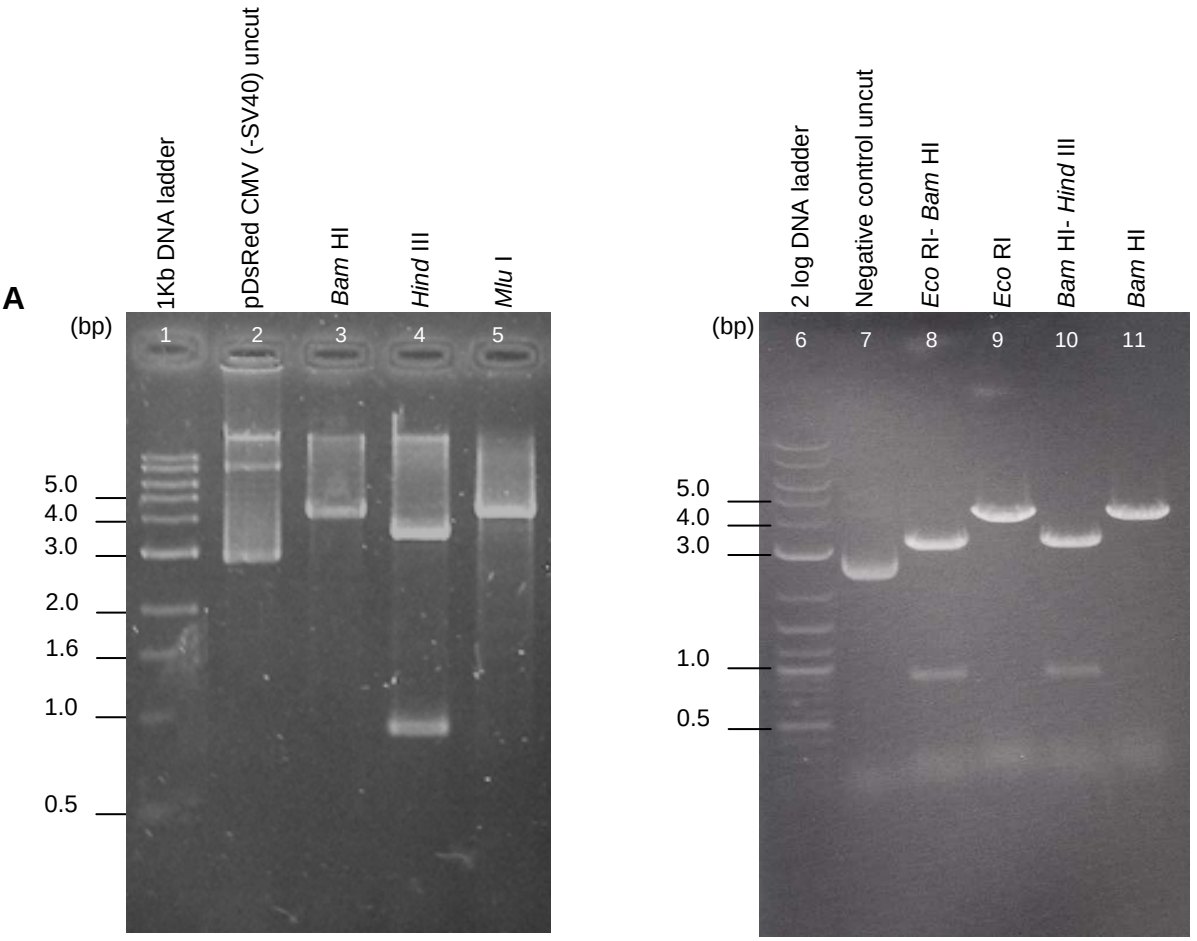
pDsRed CMV	Restriction enzyme	Expected length fragments (bp)
Positive control	*Ssp* I	4509- 553
	Stu I	3539- 1523
	Pst I	3726- 1336
	*Eco*R I- *Bam*H III	4076- 986

Figure 18: Restriction analysis of pDsRed CMV (positive control). A: Samples were electrophoresed on 1% agarose gel in TAE 1 X buffer at 70V. **B**: Restriction Map. **C**: Expected fragments length generated when the plasmid was digested with the described restriction enzymes.

- pDsRed CMV(-SV40) recombinant clone

Samples were digested with the desired restriction enzymes described in Figure 19B. The digestion of pDsRed CMV (-SV40) with *Hind* III generated a fragment of approximately 986 bp corresponding to the CMV sequence as indicated in Figure 19A lane 4. The restriction enzymes *Bam*H I*, Mlu* I and *Eco*R I opened the molecule resulting in a linear molecule of 4130 bp as shown in lanes 3, 5 and 9. Digestion with *Eco*R I - *Bam*H I*, Bam*H I- *Hind* III released the CMV sequence from this plasmid generating the 986 bp fragment (see lanes 8 and 10).

A



B

pDsRed CMV (-SV40)	Restriction enzyme	Expected length fragments (bp)
Negative control	*Bam* HI	4130
	Hind III	3156- 973
	Mlu I	4130
	Eco RI-*Bam* HI	3144- 985
	Eco RI	4130
	Bam HI- *Hind* III	3137- 973- 19

C

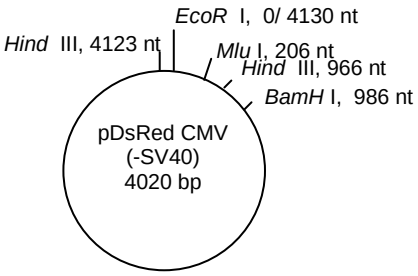
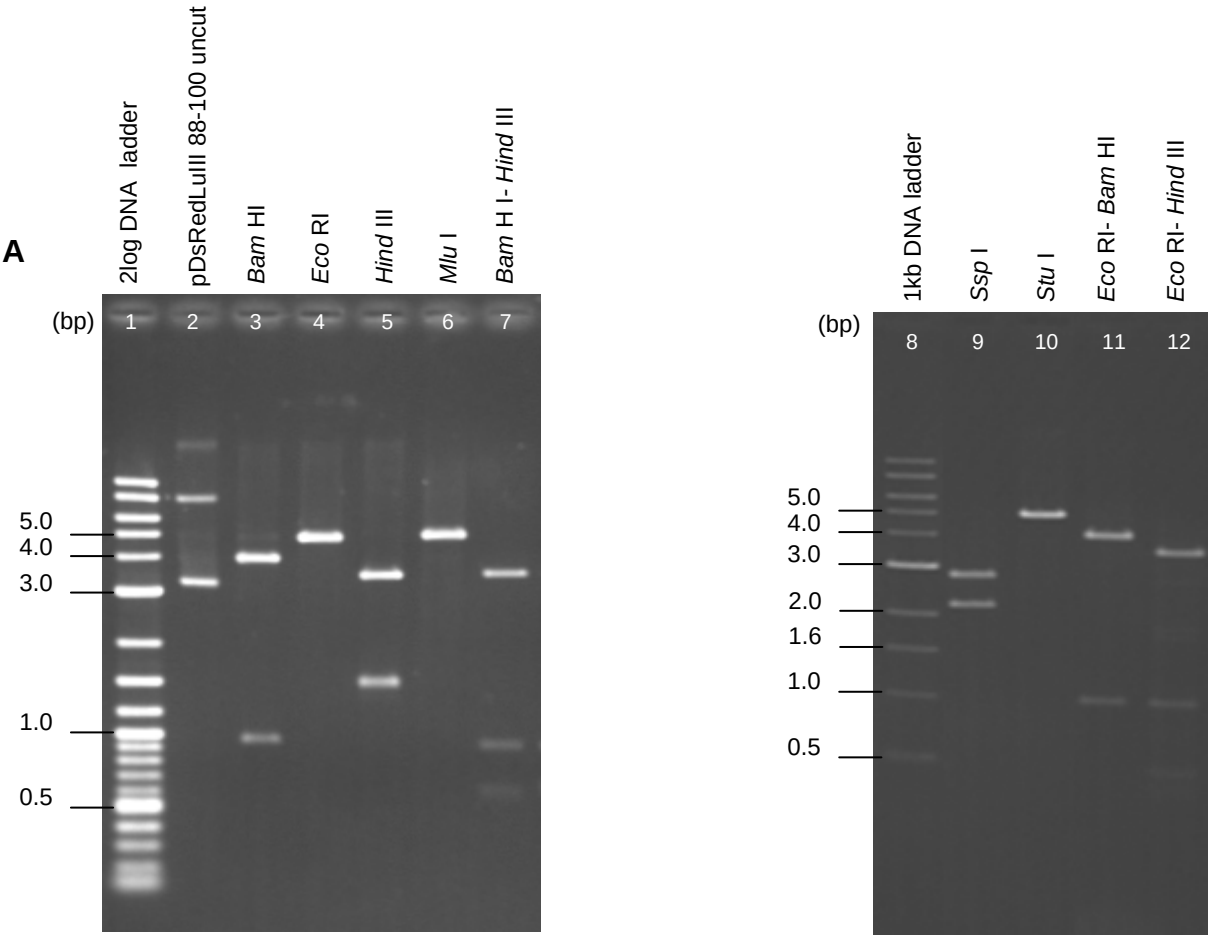


Figure 19: Restriction analysis of pDsRed CMV (-SV40) (negative control). A: Samples of pDsRed CMV (-SV40) minipreparations were digested as indicated and electrophoresed on 1% agarose gel in TAE 1 X buffer at 70V. **B**: Expected fragments generated when DNA were digested with the indicated restriction enzymes. **C**: Restriction Map.

- pDsRed LuIII 88-100 recombinant clone

The restriction enzymes used to digest the samples are indicated in Figure 20B. The pDsRed LuIII 88-100 molecule was digested with *Bam* HI releasing the CMV sequence, generating fragments of 987 bp and 3881 bp respectively, as indicated in lane 3. The enzymes *Eco* RI*, Mlu* I and *Stu* I cut once, generating a linear molecule of 4767 bp, (lanes 4, 6 and 10). *Hind* III generated two fragments, one of 1524 bp corresponding to the LuIII-CMV sequence, and other of 3165 bp that corresponds to the rest of the plasmid (lane 5). *Ssp* I cuts three times generating fragments of 2703 bp, 2188 bp and 76 bp (lane 9). Digestion with the enzymes *Bam* HI- *Hind* III and *Eco RI* - *Hind* III generated three fragments, one corresponding to the 601 bp of LuIII sequence, the CMV sequence which is 986 bp in length, and the third fragment corresponding to the rest of the molecule of approximately 3184 bp as shown in lanes 7 and 12.

A



B

pDsRed LuIII 88-100	Restriction enzyme	Expected length fragments (bp)
pDsRedLuIII 88-100	*Bam* HI	3881- 987
	Eco RI	4767
	Hind III	3165- 1524
	Mlu I	4767
	Bam HI- *Hind* III	3165- 987- 601- 14
	Ssp I	2703- 2188- 76
	Stu I	4767
	Eco RI- *Bam* HI	3780- 986- 16
	Eco RI- *Hind* III	3184- 986- 601

C

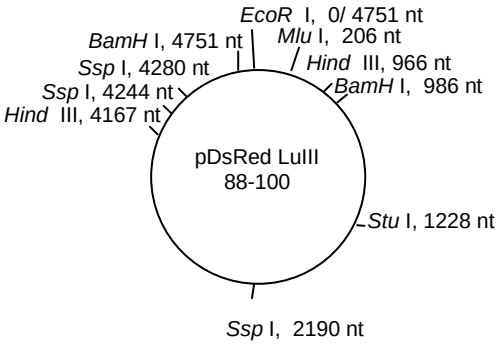


Figure 20: Restriction analysis of pDsRed LuIII88-100. **A:** Samples of pDsRedLuIII88-100 minipreparations were digested as indicated and electrophoresed on 1% agarose gel in TAE 1 X buffer at 70V. **B** Expected fragments generated when DNA was digested with the indicated restriction enzymes. **C**: restriction map

Transfection of HeLa cells and Observation in Microscope

Once the clones were characterized, one recombinant molecule of each plasmid was selected to be used for transfection of HeLa cells. HeLa cells were transfected by the electroporation method (Maxwell and Maxwell, 1988). Independent transfections with 15 ug total DNA were performed.

The reporter gene DsRed2 under the control of the CMV promoter codes for a red protein; this protein is expressed constitutively, independently of the replication origin, and normally is found throughout the cytoplasm and nucleus. Fluorescence allows us to follow the plasmid and determine indirectly if the plasmid could replicate by itself in HeLa cells. If the plasmid has the capacity to replicate, when the mother cells divide, the plasmid will be passed to the daughter cells, expressing the red fluorescent protein, resulting in an increase in the number of fluorescent cells. If the plasmid does not have the capacity to replicate by itself, the plasmid will not be passed to the daughter cells, expressing the fluorescent protein only in the mother cell and not in the daughters cells, resulting in a loss of fluorescence over time.

The cells were observed at 24, 48 and 72 hrs post-transfection with a confocal microscope, Nomarski and fluorescence interference microscope. During this time, the cells transfected with the recombinant molecules showed no obvious signs of reduced viability, and displayed the same morphology and growth characteristics as non-transfected control cells (Figure 21).

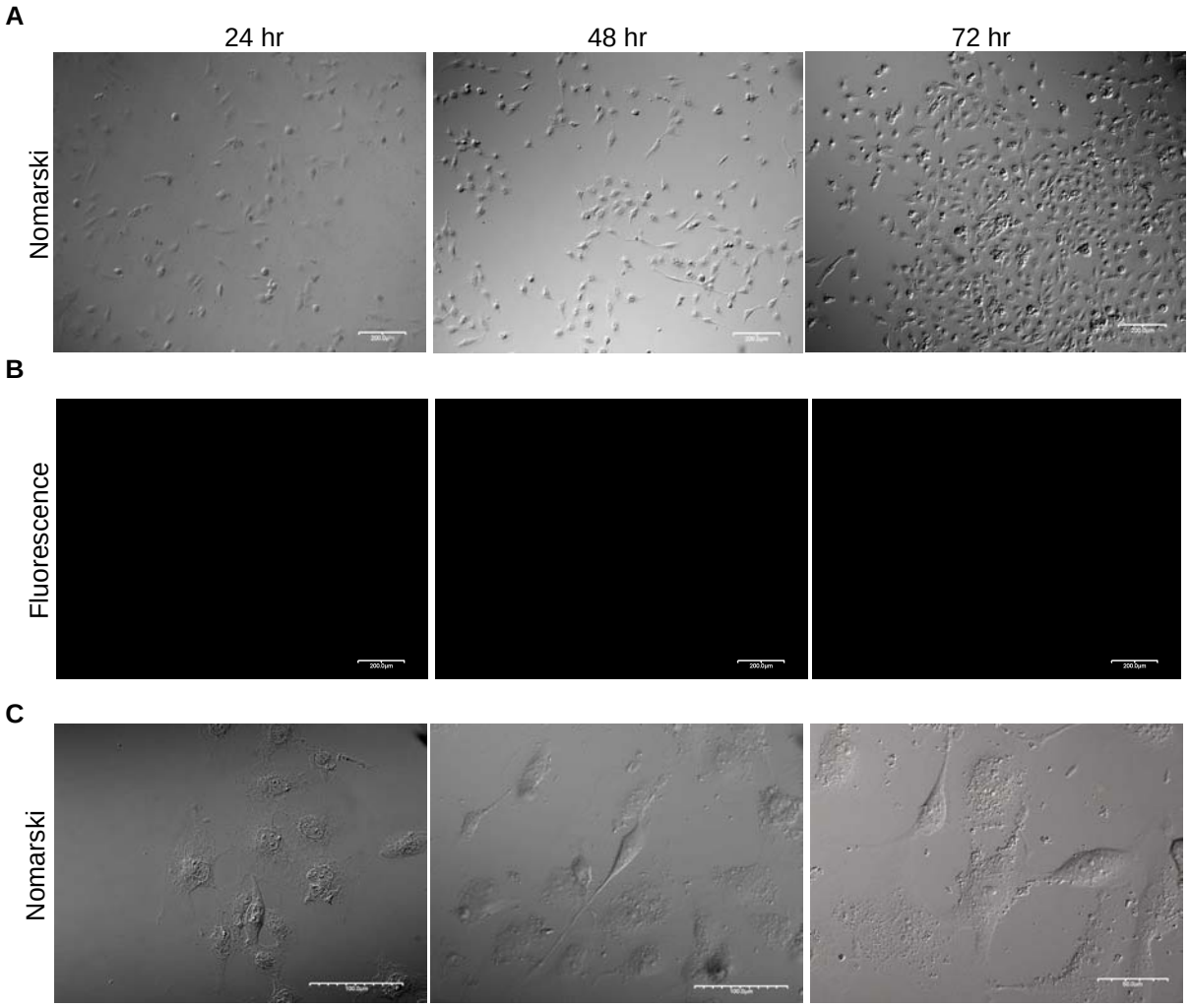


Figure 21: Non transfected HeLa cells (controls). The cells were photographed at 24, 48 and 72 hrs post-transfection in a Laser Scaning Olympus confocal microscope. **A**: Nomarski , 200.0 µm, with an objective lens of 10X. **B**: Fluorescence, in the Laser Scanning Microscope, 200.0 µm, with an objective lens of 10X. **C**: Cells were viewed at higher magnification, 40X and a digital amplification in the confocal microscope, and a scale bar of 100.0 µm, and 50.0 µm were indicated.

All the plasmids, pDsRed CMC, pDsred CMV (-SV40) and pDsRed LuIII 88-100, had the ability to express de red fluorescent protein, but only the plasmid pDsRed CMV (Figure 22) and pDsRed LuIII 88-100 (Figure 23) showed an increase in the fluorescent cell number over time. The negative control, pDsRed CMV (-SV40), had the ability to express the fluorescence protein yet this fluorescence was lost over time (personal observation, see Figure 24).

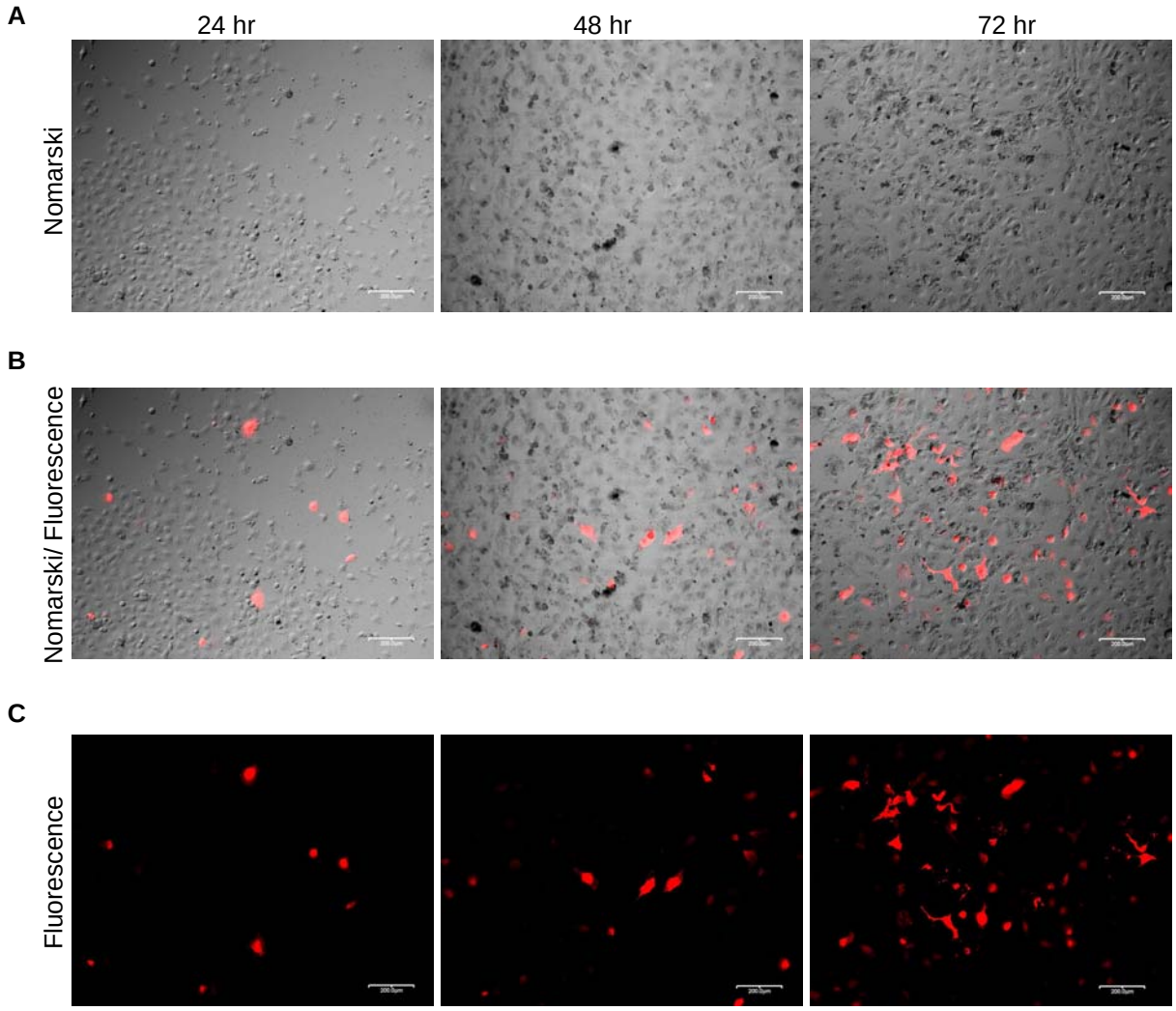


Figure 22: HeLa cells transfected with pDsRed CMV (positive control). Cells were photographed at 24, 48 and 72 hrs port-transfection in a confocal microscope. The cells were observed with a magnification of 10X objective lens, and a scale bar of 200.0 µm was used. **A**: Nomarski. **B**: Nomarski and fluorescence. **C**: Fluorescence.

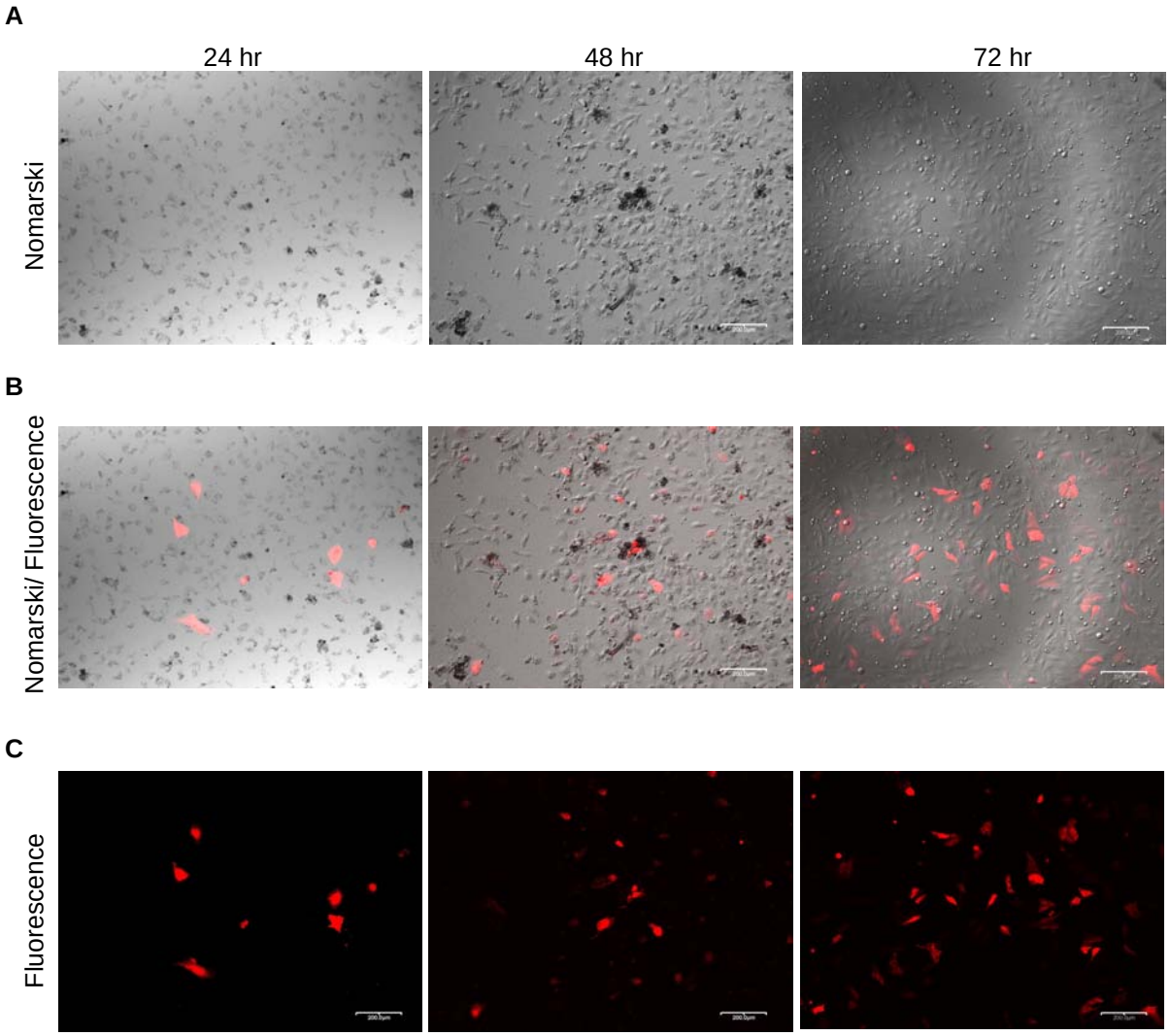


Figure 23: HeLa cells transfected with pDsRed-LuIII 88-100. Cells were photographed at 24, 48 and 72 hrs post-transfection in a confocal microscope. The cells were observed with a magnification of 10X objective lens, and a scale bar of 200.0 µm was used. **A**: Nomarski. **B**: Nomarski and fluorescence. **C**: Fluorescence.

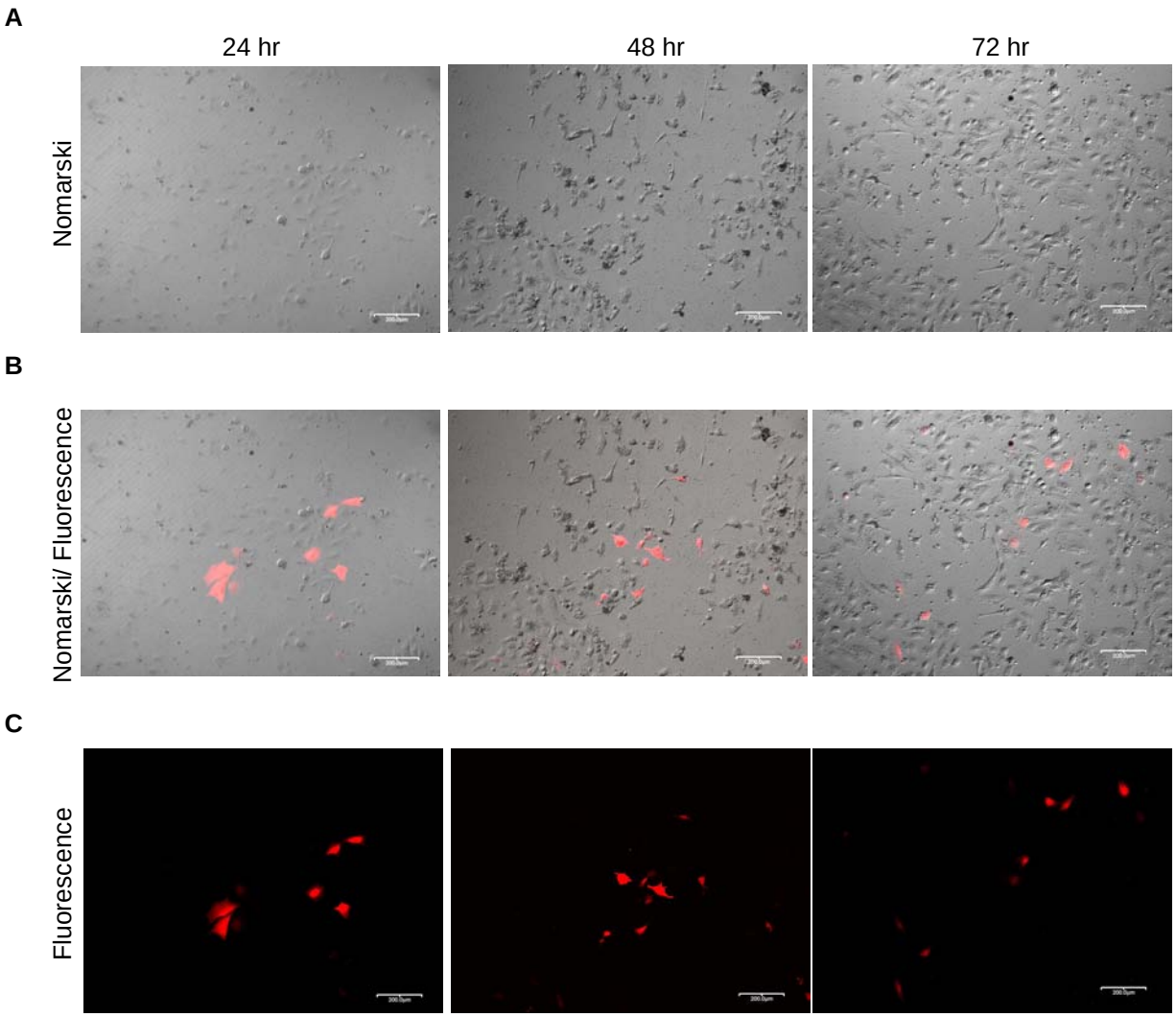


Figure 24: HeLa cells transfected with pDsRed CMV (-SV40) (negative control). Cells were photographed at 24, 48 and 72 hrs post-transfection in a confocal microscope. The cells were observed with a magnification of 10X objective lens, and a scale bar of 200.0 µm was used. **A**: Nomarski. **B**: Nomarski and fluorescence. **C**: Fluorescence.

Quantitative Analysis by Flow Cytometry

Cells were counted 72 hrs post-transfection with a flow cytometer. This technique permitted counting and sorting of the cells. Fluorescent and non-fluorescent cells were separated from a cell mixture into two fractions. For all the experiments a total of ten thousand cells were counted. The samples were done two times in duplicate each time.

The spatial distribution of cells on the flow cytometer is determined by their morphology, size and complexity, (see Figure 25A). When the fluorescence is measured, the cells with and without fluorescence are counted and separated according to the presence or absence of fluorescence, i.e. Figure 25B. The program allows the combination of more than one fluorochrome. The distribution of the cells is, in Q1: presence of fluorochomer “A” and absence of “B”, in Q2: presence of “A” and “B”, in Q3: absence of “A” and “B”, and Q4, absence of “A” and presence of “B”. Therefore, in the experiments described below, the population of red fluorescent cells occupy the bottom right square and the non-fluorescent cells are indicated in the bottom left square. In this assays the squares, Q1 and Q2 were not used because the cells only had red fluorescence and a single fluorochrome was used.

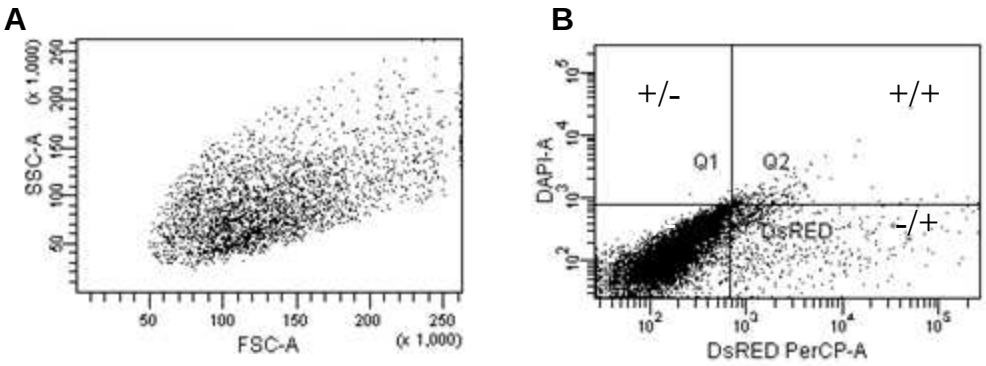


Figure 25: Example of spatial distribution of cells generated by the flow cytometer. A: total of ten thousand cells counted. A: Distribution of cells according to their size and viability. **B**: Distribution of cells according the presence or absence of the fluorescence.

Figure 26A shows the flow cytometry analyses of HeLa cells results after 72 hrs of transfection. This result indicates that morphologically the cells were in good health, according to size and complexity. The two experiments indicate that the cell distribution was as expected. The results were expressed as percentage (%), as a ratio of fluorescent cells with respect to the total cell count (Table 4 A and B). The percentage was obtained from two independent samples in the BD FACSAria Flow Cytometer (BD Biosciences). The cells were counted at 72 hrs post transfection with the flow cytometer.

Although the results indicate that the cells had low florescence, the results for both transfections were consistent. The condition and health of the recipient cells was important to obtain efficient gene transfer. In order to corroborate that the cells were healthy, they were analyzed in the Cell Viability Analyzer (Beckman Counter Analyzer).

The cells were healthy, a total of 935 cells were counted of which 663 were viable, representing a viability of 70.9%.

The total amount of DNA used is important for obtaining high transfection efficiency. However, large amounts of plasmids containing potent promoters like the CMV early promoter are toxic to cells. The recommended DNA concentration is 2.5 to 20 µg of DNA per transfection (Bio Rad Gene Pulser® Electroprotocol); the 10 µg of DNA used was within the recommended parameters.

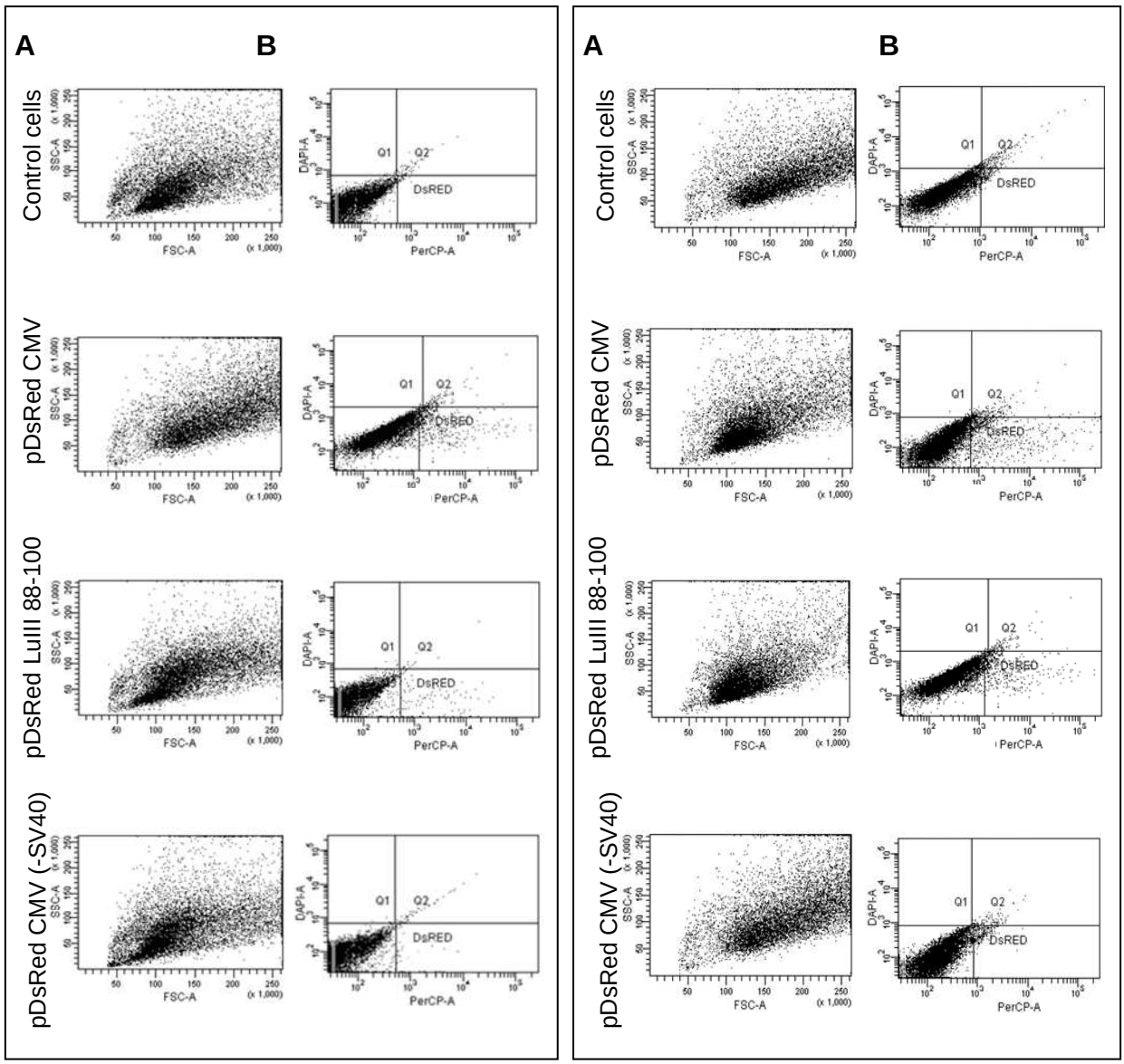
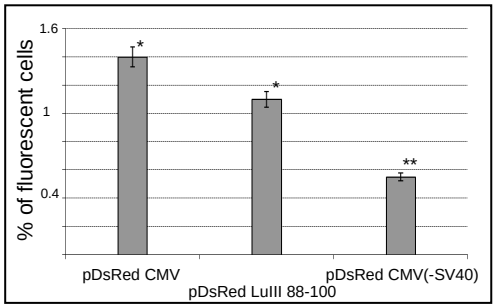


Figure 26: Spatial distribution of HeLa cell population after 72 hrs post-transfection of pDsRed CMV, pDsRed CMV (-SV40), pDsRed LuIII 88-100 plasmids. A: Distribution of cells according to their size and viability. **B**: Distribution of cells according to the presence or absence of DsRed protein.

Table 4: Percentage of Fluorescent HeLa cells after transfection with plasmids indicated and Schematic representation of the percentage of fluorescent cells. A and B represents two independent transfections each with 2 replicas, a and b, for transfection A, and, c and d, for transfection B.

A

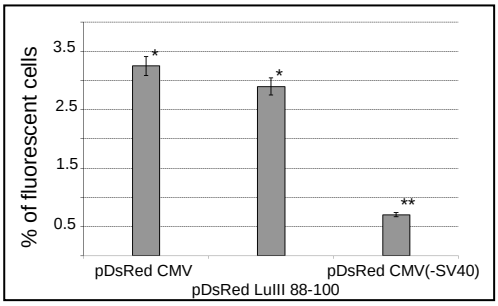
Plasmid	Cells	# cells		%	
		a	b	a	b
Cell control	NF	9913	9954	99.1	99.5
	F	33	20	0.3	0.2
pDsRed CMV Positive control	NF	9832	9815	98.5	98.2
	F	134	153	1.3	1.5
pDsRed LuIII 88-100	NF	9846	9882	98.5	98.5
	F	122	99	1.2	1
pDsRed CMV (-SV40) Negative control	NF	9885	9917	98.9	99.2
	F	62	62	0.6	0.6



NF: Non Fluorescent; F: Fluorescent;

B

Plasmid	Cells	# cells		%	
		c	d	c	d
Cell control	NF	9981	9976	99.8	99.7
	F	18	24	0.2	0.2
pDsRed CMV Positive control	NF	9508	9610	95.1	96.1
	F	338	309	3.4	3.1
pDsRed LuIII 88-100	NF	9630	9680	96.3	96.8
	F	309	268	3.1	2.7
pDsRed CMV (-SV40) Negative control	NF	9849	9884	98.5	98.8
	F	71	64	0.8	0.6



NF: Non Fluorescent; F: Fluorescent; * not significantly different, ** significantly different

When the percentage of fluorescent cells, from the different assays were compared, the number of fluorescent cells between the positive control and pDsRed LuIII 88-100 were not significantly different, (Table 4), but compared to those with the negative control, a significant difference was observed. The negative control plasmid, pDsRed CMV (-SV40) did not appear to replicate in the HeLa cells.

The results obtained from the observation of transfected cells (Figures 22, 23 and 24) with all of the plasmids show a correlation with the results obtained from the flow cytometer 72 hrs post-transfection (Table 4). The percentage of fluorescent cells, although not high (approximately 1% or 3% for the positive control), was consistent in all assays. The number of fluorescent cells observed for the sample transfected with the negative control, which does not have a replication origin, is less than the number of fluorescent cells transfected with the positive control and pDsRed LuIII 88-100 which had a SV40 replication origin or the LuIII A/T rich sequence respectively. This data suggest that the AT rich region could function as an origin of replication. The fluorescence of these cells was maintained over the observed period.

Extraction of recombinant molecules, pDsRed CMV and pDsRed LuIII 88-100, from HeLa cells, and their Bacterial Transformation into *E. coli DH5α*

If the cells were fluorescent due to the ability of the plasmid to replicate, isolation and recovery of the plasmid should be possible. To study this possibility, transfected cells with the different plasmids, pDsRed CMV, pDsRed CMV (-SV40) and pDsRed LuIII 88-100, were cultured during 6 days, and at this time, the cells were sub-cultured in a ratio 1:3. The cells were observed after 72hr post-passaged in the confocal microscope. The presence of fluorescence in all of the cells before passage indicated that the reporter gene in all of the plasmids worked properly. If the plasmid has the capacity to replicate, either due to the presence of the SV40 origin or the LuIII sequence, the plasmid should be passed from the mother to the daughter cell and consequently result

in fluorescence of the daughter cells; therewith an increase in fluorescent cell number is expected over time. If the plasmid did not have the capacity to replicate by itself, the plasmid should not be passed to the daughter cells, resulting in a decrease of fluorescence over time.

The results obtained from the observation of transfected and passed HeLa cells with pDsRed CMV which have the SV40 origin of replication, and pDsRed CMV (-SV40) which does not , show that only the plasmid pDsRed CMV and pDsRed LuIII 88-100could replicate in HeLa cells showing the red fluorescent protein in the daughter cells. The plasmid which had the LuIII A/T rich sequence also resulted in fluorescent cells after being passed suggesting that the LuIII mu 88-100 was capable of driving the replication of the plasmid (Figure 27).

This result could explain why in Figure 24 the number of fluorescent cells does not show an increase in number, and why flow cytometer percentages of fluorescent cells were lower for pDsRed CMV (-SV40), than for pDsRed CMV and pDsRed LuIII 88-100 at 72 hrs post-transfection (Figure 26).

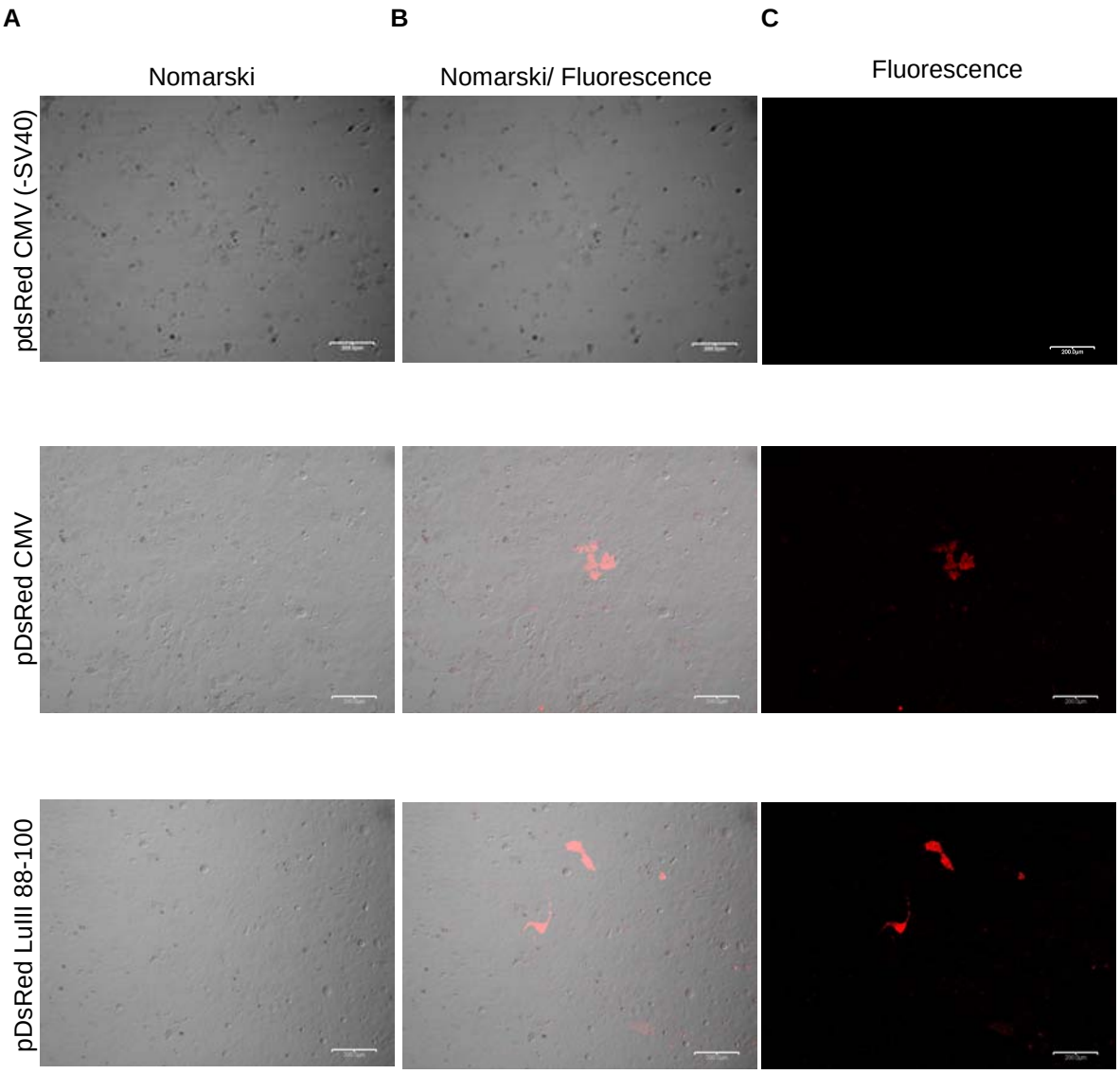


Figure 27: Observation of HeLa cells transfected with pDsRed CMV, pDsRed CMV (-SV40) amd pDsRed LuIII 88-100 at 72 hrs after being passaged. Cells transfected initially with the corresponding plasmid were photographed at 24, 48 and 72 hrs post-passaging in a confocal microscope. The cells were observed with a magnification of 10X objective lens, and a scale bar of 200.0 µm was used. **A**: Nomarski. **B**: Nomarski and fluorescence. **C**: Fluorescence.

A total DNA extraction was done by the Hirt method as described in material and methods (page 52). If the plasmid had the ability to replicate in the cells it should be rescued from the HeLa cells. Once the plasmids were isolated the DNAs were digested with *Dpn* I and *Mbo* I. These enzymes allow us to differentiate between the DNA that has been replicated and the input plasmid. Both enzymes recognize the same sequence of DNA, but *Dpn* I cuts only if the DNA is methylated, therefore this is DNA that has not replicated, whereas *Mbo* I cuts the DNA that is not methylated, therefore it has replicated. The digested and uncut DNAs were used then to transform competent *E coli DH5α* cells by the calcium chloride method.

This assay was done two times with two independent DNA extractions. The transformation efficiency of *E coli DH5α* was 1.8 x 10^6 CFU/µg DNA and 1.0 x 10^6 CFU/µg DNA. Results of the transformation are shown in Table 5. The results of the control plates for cell growth and sensibility to the antibiotics are the same as described previously. Normal growth of non transformed cells on plates without ampicillin or kanamycin, no growth on plates with antibiotics demonstrated the cells were both viable and sensitive to the antibiotics and they were not contaminated.

Table 5: *E coli DH5α* transformants with possible pDsRed CMV, pDsRed CMV (-SV40), pDsRed LuIII 88-100 clones, obtained from the transfected HeLa cells.

Sample		# of colonies [1]	# of colonies [2]
Cells control		TNTC*	TNTC*
Cells control with ampicillin		0	0
Cells control with kanamycin		0	0
Transformation control pUC 19 (10ng)		1805	4853
pDsRed CMV (positive control)	uncut	203	406
	Mbo I	52	127
	Dpn I	132	203
	Dpn I-*Mbo* I	0	0
pDsRedLuIII 88-100	uncut	10	28
	Mbo I	6	7
	Dpn I	21	13
	Dpn I-*Mbo* I	0	0
pDsRed CMV (-SV40) (negative control)	uncut	0	0
	Mbo I	0	0
	Dpn I	0	0
	Dpn I-*Mbo* I	0	0

*Too numerous to count; [1] first assay; [2] second assay.

The formation of colonies when *Dpn* I digested DNA is used to transform, indicates that the plasmid has the ability to replicate. *Dpn* I enzymes cut the methyled DNA so, the transformation resulted from the DNA that replicated in HeLa cells. The DNAs that resulted in colonies were pDsRed CMV and pDsRed LuIII 88-100. These results indicate that these plasmids had the ability to replicate in HeLa cells; on the other hand, the results obtained from the transformation with pDsRed CMV (-SV40) digested with *Dpn* I did not show the same results, indicating the absence of replicated plasmid; therewith, its inability to replicate in HeLa cells.

The presence of colonies obtained from the transformation of *E coli DH5α* with the *Mbo* I DNA digestion indicates that the input DNA was maintained in the HeLa cells. Colonies were obtained for pDsRed CMV and pDsRed LuIII 88-100 digested with *Mbo* I

suggesting that the original input DNA was maintained in HeLa cells. In the case of pDsRed CMV (-SV40) the absence of colonies indicated that this plasmid could not be maintained in the HeLa cells. The HeLa cell cycle is 20 hrs, if the plasmid could not replicate the plasmid was likely lost before the passage of the cells, therefore no fluorescence was expected.

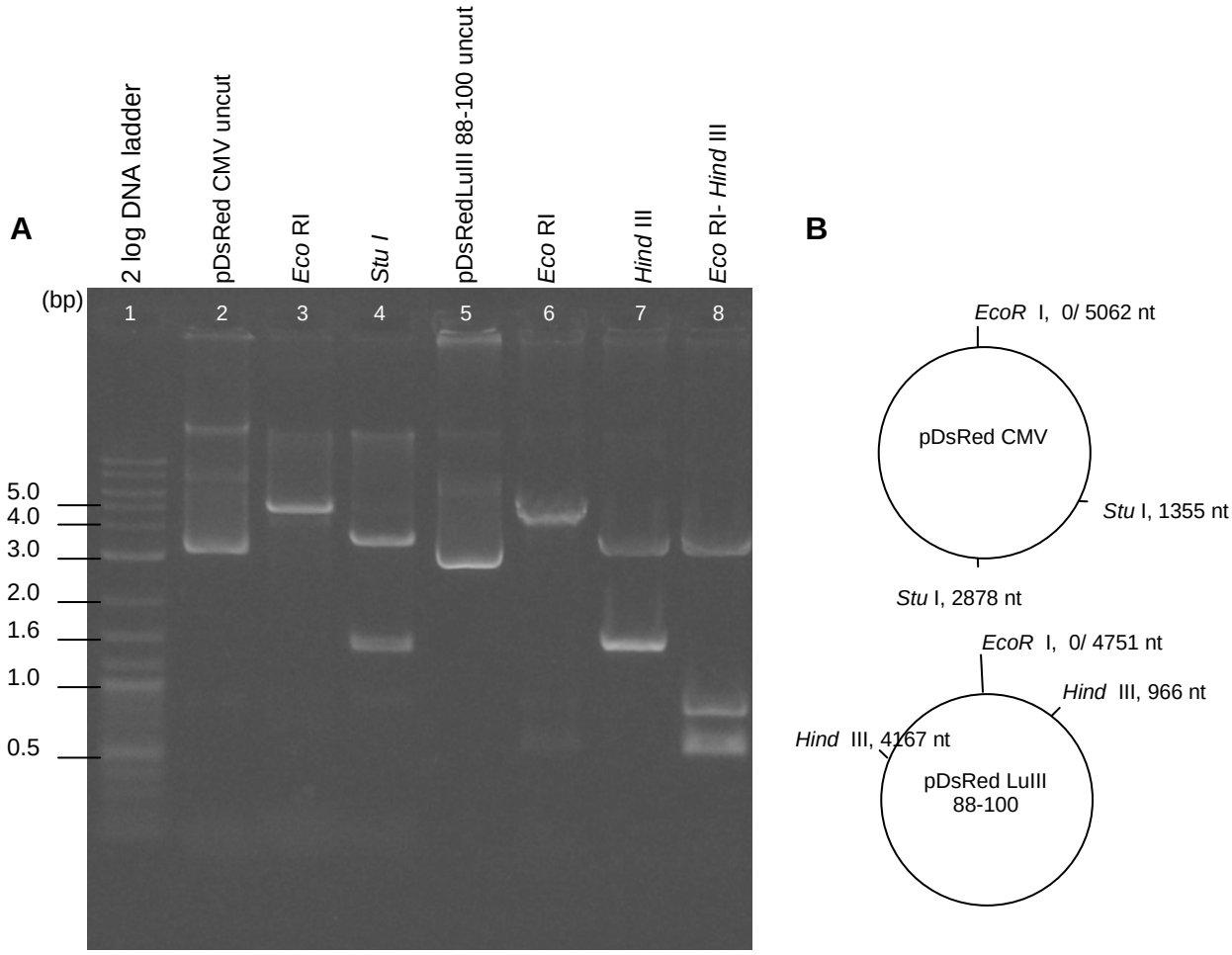
Transformation experiments with the isolated plasmids correlate with the results observed by Flow Cytometry (Figure 27); the plasmids that had the ability to replicate or to be maintained after passage of HeLa cells were pDsRed CMV and pDsRed LuIII 88-100.

Restriction Analysis of Recovered Recombinant Plasmid

The colonies obtained from the transformation with pDsRed CMV and pDsRed LuIII 88-100 plasmids were inoculated in LB broth with the corresponding antibiotic, and small scale plasmid preparation was made as described (Ausubel *et al.,* 2005).

The minipreparations were digested with the corresponding enzymes to characterize and analyze each plasmid (Figure 28). The minipreparation corresponding to the transformation of *E coli DH5α* with the pDsRed CMV was digested with *Eco* RI and *Stu* I. The restriction enzyme *Eco* RI cuts one time generating a linear molecule of 5062 bp, and *Stu* I cut two times, generating two fragments of 3539 bp and 1523 bp (lane 2-4).

The minipreparation corresponding to the transformation with pDsRed LuIII 88-100 was digested with *Eco* RI, *Hind III* and *Eco* RI-*Hind* III*.* The digestion with *Eco* RI cut between the LuIII A/T sequence and the CMV sequence and resulted in a linear molecule of 4767 bp. On the other hand, *Hind* III generated two fragments corresponding to the LuIII sequence and CMV together, 1584 bp, and the rest of the plasmid, 3184 bp. The digestion with *Eco* RI-*Hind* III *g*enerated three fragments of approximately 3184 bp, 986 bp, and 601 bp (lane 5-8).



C

Plasmid	Restriction enzyme	Expected length fragment (bp)
pDsRed CMV (Positive control)	*Eco* RI	5062
	Stu I	3539, 1523
pDsRedLuIII 88-100	*Eco* RI	4767
	Hind III	3184, 1584
	Eco RI- *Hind* III	3184- 986- 601

Figure 28: Restriction analysis of pDsRed CMV (positive control) and pDsRed LuIII 88-100 colonies obtained from transfected HeLa cells. A: Samples of pDsRed CMV and pDsRedLuIII 88-100 minipreparations were electrophoresed on 1% agarose gel in TAE 1 X buffer at 70V. **B**: Restriction map. **C**: Expected length of fragments generated when digested with the indicated restriction enzymes.

Restriction analysis of the recombinant molecules, pDsRed CMV and pDsRed LuIII 88-100, resulted in the expected restriction patterns suggesting that the recovered plasmid from transfected and passaged HeLa cells was the plasmid transfected initially.

These results demonstrate that the pDsRed LuIII 88-100 that has the A/T rich sequence was capable to replicate in HeLa cells, suggesting that the similarity observed between the ARS of yeast also correlates with a similar function at m.u 88-100 of the LuIII genome. Therefore this data support previous works that suggested a replication like origin present at mu 88-100 of parvovirus LuIII. This viral sequence may represent the origin of replication expected at the right terminus of the LuIII genome.

The Parvovirus LuIII replication model proposes a replication origin at the 3' terminus of the plus strand which is responsible for the synthesis of the minus strand of LuIII; the plus strand synthesis requires replication from the right termini of the minus strand. The identification of a replication origin at the right terminus of LuIII suggest that replication likely occurs from both strands generating the plus and minus strand with equal frequency, therewith, resulting in the encapsidation pattern observed for LuIII (50% minus strand, 50% plus strand).

CHAPTER V

Conclusions

- Three clones were constructed in this work: pDsRed CMV, pDsRed CMV (-SV40) and pDsRed LuIII 88 -100.

- The transfected HeLa cells with those plasmids were observed under microscope and all of them showed the expression of red fluorescent protein, but only the pDsRed CMV and pDsRed LuIII 88-100 showed an increase in number of fluorescent cells over time, indicating that both plasmids were capable of replication.

- The utilization of Flow Cytometry confirms that at 72 hrs post transfection the number of fluorescent cells with the plasmid pDsRed CMV and pDsRed LuIII 88-100 were greater that with the pDsRed CMV (-SV40).

- The *Dpn* I and *Mbo* I restriction analysis of DNA samples (pDsRed CMV, pDsRed CMV (-SV40) and pDsRed LuIII 88-100) recovered from HeLa transfected cells after they were subcultured confirmed the presence of replicated DNA of pDsRed CMV and pDsRed LuIII 88-100.

Therefore, this work presents evidence that the recombinant plasmid pDsRed LuIII 88-100, containing the sequence from map unit 88 - 100 (nt. 4527-5135) of parvovirus LuIII was capable of replicating in human cells demonstrating that these sequences can act as a replication origin in higher eukaryotic cells.

CHAPTER VI

Recommendations

1. Study the replication of pDsRed CMV, pDsRed CMV (-SV40) and pDsRed LuIII 88-100 during cellular division after adding the Carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDAse). With the CFSE technique, cell division could be visualized by the sequential halving of fluorescence in each daughter cell; therefore we can perform a quantitative analysis of the number of progenitor cells that enter division.

2. Study the effect of the non structural protein NS1 in the replication of pDsRed LuIII 88-100 plasmid. Given NS1 protein its evolved in viral replication, it is possible that between these sequences the presence of an active NS1 driven origin increases the replication efficiency of pDsRed LuIII 88-100 plasmid.

3. Study if the 47 bp A/T sequence is essential and necessary for the LuIII 88- 100 m.u. sequence to act as an origin of replication, by construction a plasmid pDsRed LuIII 90-100 (-A/T).

Literature cited

Arroyo, N. 2000. Possible autonomously replicating sequence (ARS) function of the unique A/ T rich nucleotide sequence of parvovirus LuIII. M.S. Thesis. University of Puerto Rico, Mayagüez, P.R.

Astell, C. R., M. Thompson, M. Merchlinsky, and D. C. Ward. 1983. The complete nucleotide sequence of Minute Virus of Mice, an autonomous parvovirus. Nucl. Acids Res. 11: 999-1018.

Astell C. R., M. B. Chow, and D. C. Ward. 1985. Sequence analysis of the termini of virion and replicative forms of Minute Virus of Mice DNA suggests a modified rolling hairpin model for autonomous parvovirus DNA replication. J. Virol. 54: 171-177.

Astell, C. R., E. M. Gardiner, and P. Tattersall, 1986. lymphotrophic variant of minute virus of mice, MVM(i), and comparison with DNA sequence of the fibrotropic protype strain. J. Virol. 57:656–669

Ausubel, F. M., B. Roger, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 2005. Current protocols in Molecular Biology. Current Protocols, Wiley, USA.

Baldauf, A. Q., K. Willwand, E. Mumtsidu, J. P. F. Nüesch and J. Rommelaere. 1997. Specific initiation of replication at the right-end telomere of the closed species of Minute Virus of Hice replicative-form DNA. J. Virol. 71:971-980.

Bates, R. C., C. E. Snyder, P. T. Bannerjee, and S. Mitra. 1984. Autonomous parvovirus LuIII encapsidates equal amounts of plus and minus DNA strands. J. Virol. 49:319–324.

Berns, K. I. 1990. Parvovirus replication. Microbiol. Rev. 54:316–329

Berns, K. I. 1996. Parvoviridae: The Viruses and Their Replication. In: B. N. Fields, D. M. Knipe and P. M. Howley (eds.). Fundamental Virology, pp. 817-833. Lippincott-Raven Publishers, Pennsylvania.

Berns, K. I. and Linden, R. M. 1995. The cryptic life style of adeno-associated virus. *Bioassays* 17, 237-245.

Blechacz B, and S.J. Russell . 2004. Parvovirus vectors: use and optimisation in cancer gene therapy. Expert Rev Mol Med. 23; 6(16):1-24.

Bossin, H., P. Fournier, C. Royer, P. Barry, P. Cerutti, S. Gimenez, P. Couble, and M. Bergoin. 2003. Junonia coenia densovirus-based vectors for stable transgene expression in Sf9 cells: influence of the densovirus sequences on genomic integration J Virol. 77(20):11060-71.

Breier A. M, Sourav C. and N R Cozzarelli. 2004. Prediction of *Saccharomyces cerevisiae* replication origins. *Genome Biology*, 5,R 22. Available at http://genomebiology.com/2004/5/4/R22

Burnett E. and P. Tattersall 2003. Reverse Genetic System for the Analysis of Parvovirus Telomeres Reveals Interactions between Transcription Factor Binding Sites in the Hairpin Stem, J. Virol. 77:8650–8660

Burnett E, F. Cotmore, and P Tattersall, 2006. Segregation of a Single Outboard Left-End Origin Is Essential for the Viability of Parvovirus Minute Virus of Mice. J. Virol, 80 (21)10879–10883

Caillet-Fauquet, P., M. Perros, A. Brandenburger, P. Spegelaere, and J. Rommelaere. 1990. Programmed killing of human cells by means of an inducible clone of parvoviral genes encoding non-structural proteins. EMBO J. 9:2989-2995.

Chen, K.C., J.J. Tyson, M. Lederman, E.R. Stout, and R.C. Bates. 1986. A kinetic hairpin transfer model for parvoviral DNA replication. J. Molec. Biol. 208:283-296

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

Christensen J. and P. Tatersall, 2002. Parvovirirus initiator protein NS1 and RPA coordinate replication fork progression in a reconstituted DNA replication system. J Virol. 76:6518-6531.

Cornelis, J.J., N. Salome, C. Dinsart, J. Rommelaere. 2004. Vectors based on autonomous parvoviruses: novel tools to treat cancer? J Gene Med. 6 Suppl 1:S193-202.

Corsini J, Carlson J.O, Maxwell F, Maxwell I.H. 1995. Symmetric-strand packaging of recombinant parvovirus LuIII genomes that retain only the terminal regions. J Virol. 69(4):2692-6.

Corsini J., I.H. Maxwell, F. Maxwell, and J.O. Carlson. 1996. Expression of parvovirus LuIII NS1 from a Sindbis replicon for production of LuIII-luciferase transducing virus. Virus Res. 46(1-2):95-104

Cossart, Y. E., Field, A. M., Cant, B. and Widdows, D. 1975. Parvovirus-like particles in human sera. Lancet , 72–73.

Cotmore S. F. and P. Tattersal. 1995. DNA replication in the autonomous parvoviruses. Semin. Virol. 6: 271-281

Cotmore, S. F. and P. Tattersall. 1987. The autonomously replicating parvoviruses of vertebrates. Adv. Virus Res. 33: 91-173.

Cotmore S. F. and P. Tattersall. 1988. The NS-1 polypeptide of Minute Virus of Mice is covalently attached to the 5' termini of duplex replicative-form DNA and progeny single strands. J. Virol. 62: 851-860.

Cotmore S.F. and P. Tattersall. 1990. Alternate splicing in a parvoviral nonstructural gene links a common amino-terminal sequence to downstream domains, which confer radically different localization and turnover characteristics. Virol. 177: 477-487.

Cotmore, S. F. and P. Tattersall. 1994. An asymmetric nucleotide in the parvoviral 3' hairpin directs segregation of a single active origin of DNA replication. EMBO J. 13: 4145-4152.

Cotmore S. F. and P. Tattersal. 1995. DNA replication in the autonomous parvoviruses. Semin. Virol. 6: 271-281

Cotmore, S. F. and P. Tattersall. 1998. High-mobility group 1/2 proteins are essential for initiating rolling-circle-type DNA replication at a parvovirus hairpin origin. J. Virol. 72: 8477-8484.

Cotmore S. F. and P. Tattersall. 2003. Resolution of parvovirus dimer junctions proceeds through a novel heterocruciform intermediate. J. Virol. 77 (11): 6245-6254.

Cotmore S. F. and P. Tattersall. 2005. Encapsidation of minute virus of mice DNA: Aspects of the translocation mechanism revealed by the structure of partially packaged genomes. Virol. 336 100–112

Cotmore, S. F., Gunther, M., and P. Tattersall. 1989 Evidence for a ligation step in the DNA replication of the autonomous parvovirus minute virus of mice *J. Virol.* **63,** 1002–1006. *J. Virol.* 63**,** 1002–1006

Cotmore S., J. Christensen, and P. Tattersall. 2000. Two widely spaced initiator binding sites create an HMG1-dependent parvovirus rolling-hairpin replication origin. J. Virol. 74: 1332-1341

Cotmore S. F., A. J. Dabramo, L. F. Carbonell, J. Bratton, and P. Tattersall. 1997. The NS2 polypeptide of parvovirus MVM is required for capsid assembly in murine cells. Virol. 231: 267-280.

De Jesús-Maldonado, I. 2004. Study of an ARS-like function of map units 88-100 of Parvovirus LuIII. Thesis M.S. Universidad de Puerto Rico, Mayagüez, PR.

Diffoot N., B.C. Shull, K.C. Chen, E.R. Stout, M. Lederman, and R.C. Bates. 1989. Identical ends are not required for the equal encapsidation of plus- and minus-strand parvovirus LuIII DNA. J Virol. 63(7):3180-4

Diffoot N., K.C. Chen, R.C. Bates, and M. Lederman. 1993. The complete nucleotide sequence of parvovirus LuIII and localization of a unique sequence possibly responsible for its encapsidation pattern. Virol. 192(1):339-45.

Diffoot-Carlo N., L. Vélez-Pérez, and I. De Jesús-Maldonado. 2005. Posible active origin of replication in the double stranded extended formo f the left terminus of LuIII end its implication on the replication model of the parvovirus. J. Virol. 31;2:47.

Dumas B., M. Jourdan, A.M. Pascaud, and M. Bergoin. 1992. Complete nucleotide sequence of the cloned infectious genome of Junonia coenia densovirus reveals an organization unique among parvoviruses. Virol. 191(1):202-22.

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

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

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

Faisst, S., J. Rommelaere (eds). 2000. Parvoiruses. From Molecular Biology to Pathology and Therapeutic Uses. Contrib. Microbiol. Basel, Karger. Vol 4: 163-202.

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

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

Heegaard, E.D., and A. Hornsleth . 1995. Parvovirus: the expanding spectrum of disease. Acta Paediatr. 84(2):109-17.

Im D.S. and N. Muzyczka. 1990. The AAV origin binding protein Rep68 is an ATP-dependent site-specific endonuclease with DNA Helicase activity. Cell. 61: 447-457.

Kings JA, R. Dubielzig, D. Grimm, and J.A. Kleinschmidt. 2001. DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. EMBO J. 15; 20(12): 3282-3291.

Kotin, R. M., Siniscalco, M., Samulski, R. J., Zhu, X. D., Hunter, L., Laughlin, C. A., McLaughlin, S., Muzyczka, N., Rocchi, M. and K. I Berns. 1990. Site-specific integration by adeno-associated virus. Proceedings of the National Academy of Sciences of the U.S.A. 87: 2211-2215.

Kufe DW, S. Advani, and R. Weichselbaum. Principles of gene therapy. In: Bast, Kufe, Pollock, Weichselbaum, Holland, Frei, eds. *"Cancer Medicine".* Hamilton, Ontario: BC Decker Publisher, 2000. 876-890

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

Labow, M. A., L. H. Graf, Jr., and K. I. Berns. 1987. Adeno-associated virus gene expression inhibits celullar transformation by heterologous genes. Mol. cell. Biol.7: 1320- 1325.

Lombardo, E., J. Ramírez, J. García and J. Almendral. 2002. Complementary Roles of Multiple Nuclear Targeting Signals in the Capsid Proteins of the Parvovirus Minute Virus of Mice during Assembly and Onset of Infection. J. Virol. 76:7049-7059.

Lukashov V.V. and J. Gouudsmit. 2001. Evolutionary Relationships among Parvoviruses: Virus-Host Coevolution among Autonomous Primate Parvoviruses and Links between Adeno-Associated and Avian Parvoviruses. J. Virol. 75: 2729-2740.

Lusby, E., R. Bohenzy and K.I. Berns. 1980. Nucleotide sequence of the inverted terminal repetition in adeno-associated virus DNA. J. Virol. 34(2):402-9.

Maxwell, I.H. and F. Maxwell. 1988, Electroporation of mammalian cells with a firefly luciferase expression plasmid: kinetics of transcient expression differ markedly among cell types. DNA 7, 557-562.

Maxwell, I.H. and F. Maxwell. 2004. Parvovirus LuIII transducing vectors packaged by LuIII versus FPV capsid proteins: the VP1 N-terminal region is not a major determinant of human cell permissiveness. J Gen Virol. 85(Pt 5):1251-7.

Maxwell I.H., K.L.Terrell, and F. Maxwell. 2002. Autonomous parvovirus vectors. Methods. 28(2):168-81. Review.

Maxwell IH, Spitzer AL, Maxwell F, Pintel DJ. 1995. The capsid determinant of fibrotropism for the MVMp strain of minute virus of mice functions via VP2 and not VP1. J Virol. 69(9):5829-32.

McKenna, R., N. Olson, P. Chipman, T. Baker, T. Booth, J. Christensen, B. Aasted, J. Fox, M. Bloom, J. Wolfinbanger and M. Agbandje-McKenna. 1999. Three-Dimensional Structure of Aleutian Mink Disease Parvovirus: Implications for Disease Pathogenicity. Virol. 73:6882-6891.

Muller, D.E., and G. Siegl. 1983. Maturation of Parvovirus LuIII in a subcellular system. I. Optimal conditions for in vitro synthesis and encapsidation of viral DNA. J. Gen. Virol. 64: 1043-1054.

Naeger, L.K., N. Salome, and D. J. Pintel. 1993. NS2 is required for efficient translation of viral mRNA in minute virus of mice-infected murine cells. J. Virol. 67: 1034-1043

Newlon, C. S. 1988. Yeast chromosome replication and segregation. Microbiol. Rev. 52:568-601.

Palzkill, T.G and C.S Newlon. 1998. A yeast replication origin consist of multiple copies of a small conserved sequence. Cell 6; 53: 441-50.

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

Qiu, J., F. Cheng, F.B. Johnson, and D. Pintel. 2007. The transcription profile of Bocavirus Bovine Parvovirus is unlike those of previously characterized parvoviruses. J. Virol 81(21): 12080- 12085.

Rabinowitz J.E. and R. J. Samulski, 2000. Building a Better Vector: The Manipulation of AAV Virions. Virol. 278:2 (301-308

Rao, H., Y. Marahrens and B. Stillman. 1994. Functional conservation of multiple elements in yeast chromosomal replicators. Mol.Cell. Biol. 14: 7643-765

Román-Pérez, E. 2000. Replication of bacterial plasmid containing LuIII A/T rich flanking sequences in Newborn Human Embryonic Kidney cells. Thesis M.S. Universidad de Puerto Rico, Mayagüez, PR.

Shade, R., M. C. Blundell, S. F. Cotmore, P. Tattersall, and C. R. Astell. 1986. Nucleotide sequence and genome organization of human parvovirus B19 isolated from the serum of a child during aplastic crisis. J. Virol. 58: 921-936.

Siegl, G. 1984. Biology and pathogenicity of autonomous parvoviruses, p. 297 348. In K. I. Berns (ed.), The parvoviruses. Plenum Press, New York, N.Y.

Soike, K., M. Latroupolis and G. Siegl. 1976. Infection of Newborn and Fetal hamsters Induced by Inoculation of LuIII Parvovirus. Arch. Virol. 51:235-241.

Strachan Tom and Andrew Read, Human Molecular genetics, 2nd edition. New York and London: Garland Science; 1999. chapter 22

Tam P., and C. R. Astell. 1993. Replication of Minute Virus of Mice minigenomes: novel replication elements required for MVM DNA replication. Virol. 193: 812-824.

Tam P., and C. R. Astell. 1994. Multiple cellular factors bind to *cis*-regulatory elements found inboard of the 5' palindrome of Minute Virus of Mice. J. Virol. 68: 2840-2848.

Theis, J.F. and C.S. Newlon. 1997. The ARS309 replicator of S. cerevisiae depends upon an exceptional ARS consensus sequence. Proc. Nat. Acad. Sci. USA 94:10786-10791.

Theis, J.F., C. Yang, C. B. Schaefer and C. S. Newlon. 1999. DNA sequence and functional analysis of homologous ARS elements of Saccharomyces cerevisiae and S. carlsbergensis. *Genetics* 152(3):943-52

Tullis G.E, Burger, L.R, Pintel, D.J. 1993. The minor capsid protein VP1 of the autonomous parvovirus minute virus of mice is dispensable for encapsidation of progeny single-stranded DNA but is required for infectivity, J Virol. 67(1):131-41.

Van Houten J.V, and C.S. Newlon. 1990. Mutational analysis of the consensus sequence of a replication origin from yeast chromosome III. Mol Cell Biol. 10(8):3917-25.

Van Regenmortel, M. H. V., C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wickner (eds.) 2000. Virus taxonomy: classification and nomenclature of viruses. Academic Press, San Diego, Califormia.

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

Willwand, K., and B. Hirt. 1993. The major capsid protein VP2 of minute virus of mice can form particles which bind to the 39-terminal hairpin of replicative-form DNA and package single-strand viral progeny. J. Virol.67:5660–5663

Wong, J.R. and C. Saswati. 2002. Parvovirus Vectors for the Gene Therapy of Cancer. Gene Therapy of Cancer, Second Edition, chapter 3,

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

Yukiko O., H. Satoh, M. Sekiguchi, and H. Masukata. 1999. Clustered Adenine/Thymine Stretches Are Essential for Function of a Fission Yeast Replication Origin. Mol. Cel. Biol. 19 (10): 6699–6709

Zadori, Z., J. Szelei, M.C. Lacoste, S. Gariepy, P. Raymond, M. Allaire, I.R. Nabi, and P. Tijssen. 2001. A viral phospholipase A2 is required for parvovirus infectivity. Dev. Cell 1:291- 302

Zadori, Z., J. Szelei, and P. Tijssen. 2005. SAT: a late NS protein of porcine parvovirus. J. Virol. 79 (20): 13129- 13138.