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Fine Mapping of Sequences Important For FIV RNA Packaging and Their Mechanism of Function

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United Arab Emirates University
Deanship of Graduate Studies
M.Sc. Program in Environmental Science

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By

Akela Ahmed Mohamed Ghazawi

A thesis
Submitted to

United Arab Emirates University
In partial fulfillment of the requirements
For the Degree of M.Sc. in Environmental Sciences

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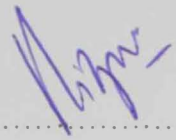
The Thesis of Akela Ahmed Ghazawi for the Degree of Master of Science in Environmental is approved.



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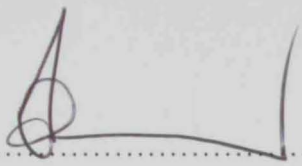

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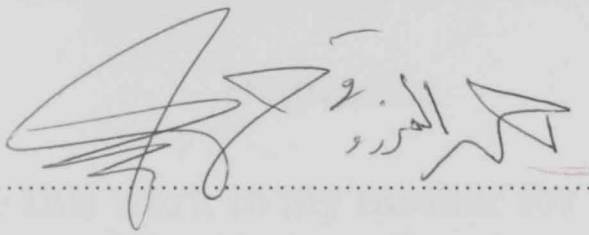

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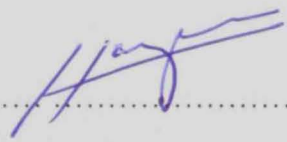

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Dedication

I dedicate this work to my mother for her unlimited giving, love and support

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Abstract

The mechanism by which retroviruses preferentially encapsidate their unspliced genomic RNA among millions of both spliced viral and cellular mRNAs in the cytoplasm represents a function of great specificity. This selection process requires that the genomic RNA contain packaging determinants unique to its own RNA that can interact specifically with the packaging proteins of the viral particles, the Gag polyproteins. Knowing the exact sequences involved in packaging should provide basic insights into the mechanism of preferentially encapsidating the full length genomic RNA.

We have been interested in mapping the packaging determinants of the feline immunodeficiency virus (FIV), a lentivirus that is being considered as a potentially powerful gene delivery system for human gene therapy. Our initial studies have shown that the FIV packaging determinants are located as two discontinuous core regions within the 5' end of the viral genome (Browning et al., 2003 a & b). The first region extends from the R/U5 in the 5' LTR to the first 120 bp of 5' UTR and the second consists of the first 100 bp of *gag*, while other regions of the genome may also be involved.

Studies undertaken in this thesis carried these observations further to determine whether the region in between the two core determinants was important for packaging or merely acted to maintain the spacing of the two core elements. Additionally, since other regions of the genome, especially the LTR, had been implicated as containing significant packaging determinants in other studies (Kemler et al., 2002), we dissected the role of the LTR elements away from the untranslated region towards FIV RNA packaging. Towards this end, several series of small FIV transfer vectors were constructed either in the heterologous non-viral or homologous subgenomic context containing various

combinations of the LTR, and/or UTR and *gag* and tested for their packaging potential in our well-established *in vivo* packaging assay. This was followed by analysis of the amount of transfer vector RNAs packaged directly into the virus particles using a semi-quantitative RT-PCR approach.

Test of the various transfer vector RNAs confirmed our earlier observation that the FIV packaging determinants are indeed discontinuous and spread out with the core packaging determinants residing within the first 150 bp UTR and 100 bp of *gag* (Chapter III). Furthermore, the intervening sequences between these two elements were not required either for vector RNA packaging or propagation (Chapter III). Analysis of the LTR elements revealed the presence of other packaging determinants of lesser strength than the core determinants in the 5' R/U5 and 3' U3/R regions of the viral LTRs (Chapter IV). Folding of the 5'end of the viral RNA using computer analysis software revealed the presence of complex stem loop structures. Correlation of the mutational analysis with the folding algorithms revealed the presence of a conserved stem loop in the 5'UTR that may serve as the principal packaging determinant of FIV. Interestingly, no consistent structural element could be identified within the first 100 bp of *gag* that could be responsible for the packaging potential of the *gag* region, suggesting that *gag* sequences may function at the primary sequence level, perhaps providing the intronic sequences needed to distinguish between genomic and subgenomic mRNAs. Taken together, these data should add to the increasing knowledge of how complex retroviruses package their genomic RNAs and help streamline the design of safer self-inactivating FIV-based vectors for human gene therapy.

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Chapter I

INTRODUCTION

Background

The technological revolution in molecular biology has brought about new ways of controlling human diseases such as gene therapy. Using genes to treat, prevent and control human diseases has improved the field of biomedical research and could further enhance the quality of millions of lives. Retroviruses are a special type of viruses that have proven to be ideal for human therapy due to their ability to integrate and stably transmit the desired genetic material to the treated cells.

In addition to medicine, the study of retroviruses has had a great impact on other areas of biology, especially on molecular genetics, on the study of cellular growth control and carcinogenesis, and overall, on biotechnology (Varmus 1988; Temin, 1992). The family *Retroviridae* includes a wide variety of disease-producing animal viruses that contain RNA as their genetic material in the virus particle and DNA as their genetic material in cells. Hence, this is the first example of a "reverse" flow of information from RNA to DNA, challenging the central dogma of biology. This is due to the presence of "reverse transcriptase" an enzyme whose main role is to convert the genomic RNA into DNA. Because of this reversal of information, these viruses are called "retro". All retroviruses and their relatives are obligatory parasites (reviewed in Vogt, 1997).

Structure

All retroviruses have a similar structure, although they cause different diseases (Fig I.1). Starting from outside, the viral envelope is formed from the host cell membrane and consists of two viral protein subunits: the transmembrane protein (TM), which is linked

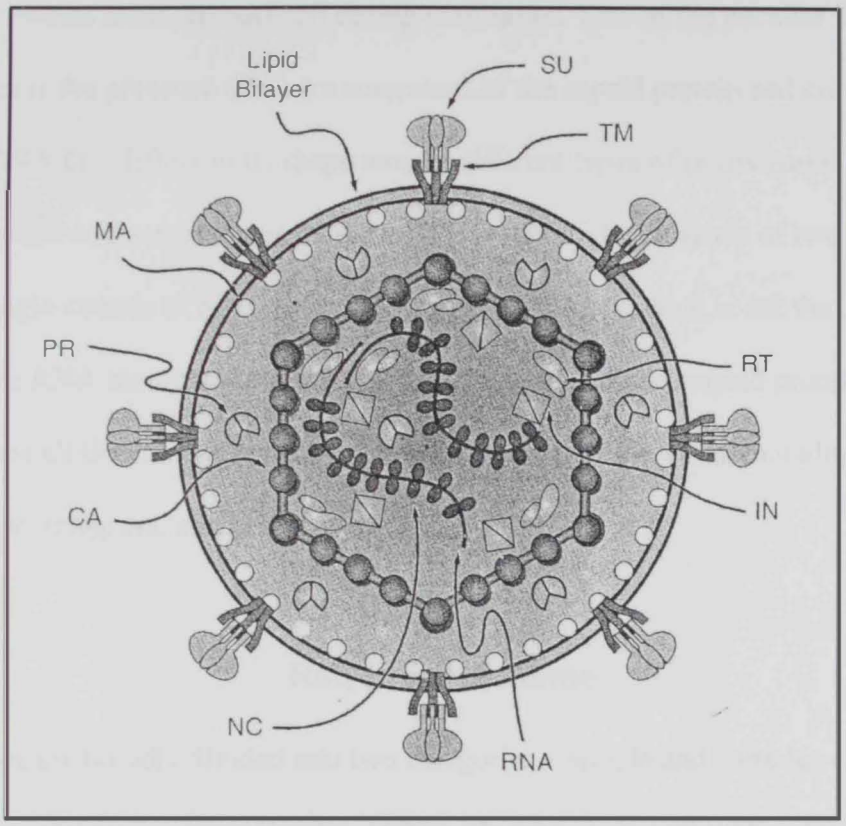

Figure I.1. Structure of a typical retrovirus. The RNA is shown in the center of the capsid (CA) in association with the nucleocapsid proteins (NC). The integrase (IN), reverse transcriptase (RT), and the protease (PR) enzymes are found within the capsid forming the inner core of the virus. Both the transmembrane (TM) and the surface (SU) envelope glycoproteins are found within the host cell membrane that is attached to the core via the matrix (MA) proteins.

[adapted from *Retroviruses*, 1997]

to the surface protein (SU). The envelope proteins are responsible for the infectious properties of the retroviruses and without them, the virus cannot enter the host cell. In addition, the virus membrane contains most of the host proteins, revealing that virus membrane comes from the host cell during maturation. One of the peculiar feature of retroviruses is the presence of a core composed of the capsid protein and containing the genomic RNA that differs in its shape among different types of retroviruses. The capsid is held together to the membrane by the matrix protein. The presence of two identical positive single-strands of RNA makes retroviruses diploid, which is not the case in other viruses. The RNA molecules are strongly attached to the nucleocapsid protein. The core also contains all the enzymes required for the life cycle of the virus, including the reverse transcriptase, integrase, and protease (reviewed in Vogt, 1997).

Retroviral Genome

Retroviruses are broadly divided into two categories – simple and complex- based on the organization of their genomes (Coffin, 1992a; Murphy et al., 1994) (Fig. I.2). While simple retroviruses contain only the structural genes necessary for virus particle formation, complex ones contain additional genes that give them further capabilities to exploit the host cellular machinery.

All retroviruses include three to four structural genes containing the information needed for the formation of new viral particles. The *gag* gene is responsible for the synthesis of structural proteins which differentiates into the matrix (MA), the capsid (CA) and the nucleocapsid (NC) proteins. The *pro* encodes for the protease enzyme (PR) that cleaves the viral polyproteins (reviewed in Vogt, 1997).

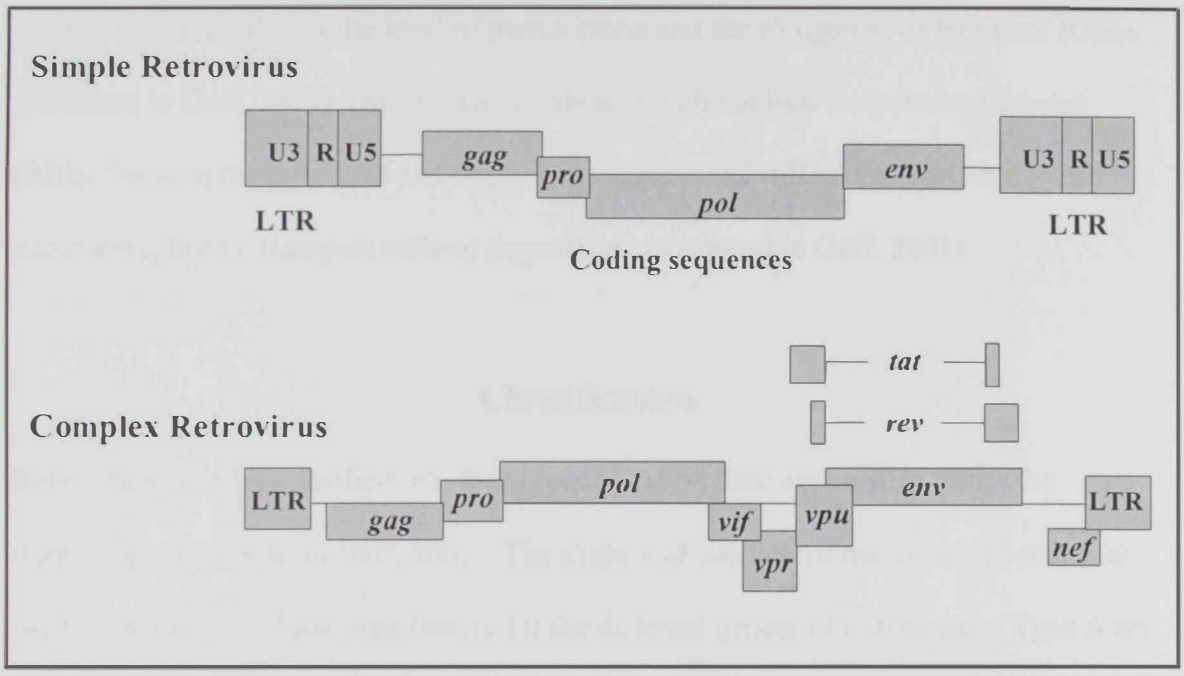

Figure 1.2. Schematic representation of simple and complex retroviruses. Genes which are common to all retroviruses include *gag, pro, pol,* and *env*. Complex retroviruses include additional genes such as *vif, vpr, vpu, nef, tat, and rev.* The non-coding sequences include the long terminal repeat (LTR) which contains the U3, R, and U5.

The *pol* encodes for enzymes critical for virus replication, including the reverse transcriptase (RT) and integrase (IN) enzymes. Finally, the product of the *env* gene is responsible for the infection of cells, forming the surface (SU) and transmembrane (TM) components of the envelope protein. The complex retroviruses include some additional genes such as *tat, rev, vif, vpr, vpu,* or *nef.* Some of these are critical for virus replication, such as *tat* which affects the level of transcription and the elongation of lentiviral RNAs (reviewed in Goff, 2001) and *rev,* which interacts with the Rev Responsive Element (RRE) found in the unspliced and singly spliced retroviral mRNAs to facilitate their nucleocytoplasmic transport without degradation (reviewed in Goff, 2001).

Classification

Retroviruses can be classified into four types based on their appearance under the microscope (reviewed in Goff, 2001). The shape and position of nucleocapsid core was used primarily as a classifying feature for the different genera of retroviruses. Type A are incomplete virions since they do not have an extracellular phase in their life cycle. That is why they are called "retrotransposons". Type B are assembled by budding and get matured with a condensed non-central core. Type C are represented in mammalian and avian groups and differ from the previous types in that they mature into a condensed central form. Finally, type D particles are very similar to type B in assembly and budding.

The above classification has been partially useful in defining retroviruses. However, a new classification was made to differentiate between the different genera of retroviruses (Goff, 2001). The new classification defines seven groups of retroviruses (Table I.1).

Table I.1: Retrovirus genera according to the new classification

New name	Examples	Morphology
Alpharetrovirus, α	Avian leukosis virus (ALV) Rous sarcoma virus (RSV)	C- type
Betaretrovirus, β	Mouse mammary tumor virus (MMTV) Mason-Pfizer monkey virus (MPMV) Jaagsiekte sheep retrovirus (JSRV)	B-, D-type
Gammaretrovirus, γ	Murine leukemia viruses (MuLV) Feline leukemia virus (FeLV) Gibbon ape leukemia virus (GaLV) Reticuloendotheliosis virus (Revt)	C-type
Deltaretrovirus, δ	Human T-lymphotropic virus (HTLV)-1, -2 Bovine leukemia virus (BLV) Simian T-lymphotropic virus (STLV)-1, -2, -3	-
Epsilonretrovirus, ε	Walleye dermal sarcoma virus Walleye epidermal hyperplasia virus 1	-
Lentivirus	Human immunodeficiency virus type 1 (HIV-1) HIV-2 Simian immunodeficiency virus (SIV) Equine infectious anemia virus (EIAV) Feline immunodeficiency virus (FIV) Caprine arthritis encephalitis virus (CAEV) Visna/maedi virus	Rod/cone core
Spumavirus	Human foamy virus (HFV)	Immature

Alpharetroviruses are simple retroviruses having a C-type morphology. The best known example of this group is Avian Leukemia Viruses (ALV). Betaretroviruses are also simple retroviruses having either a B-type morphology with an eccentric core or D-type morphology with a cylindrical core. Mouse Mammary Tumor Virus (MMTV) and Mason-Pfizer Monkey Virus (MPMV) exemplify this group. Gammaretroviruses are simple retroviruses characterized by a C-type morphology. This group has a wide variety of members, including the murine and feline leukemia viruses (MuLVs and FeLVs). Deltaretroviruses are complex retroviruses having a C-type morphology. They are exemplified by the human T-lymphotropic virus (HTLV) and the bovine leukemia viruses (BLV). HTLV-1 and 2 are an important category of retroviruses with oncogenic properties in humans. Epsilonretroviruses are complex viruses characterized by a C-type morphology. The prototype of this group is the walleye dermal sarcoma virus (WDSV). Lentiviruses are complex viruses having a unique morphology with a core that is either cylindrical or conical in shape. One of the best known examples of this group are the human immunodeficiency virus types 1 and 2 (HIV-1 and 2). This category causes neurological and immunological diseases such as acquired immunodeficiency syndrome (AIDS). Spumaviruses are complex viruses having a unique morphological feature. They contain spikes on the surface and have a central uncondensed core. The prototype of this group is the human foamy virus (HFV) (reviewed in Goff, 2001).

Retrovirus Replication

The mechanism by which retroviruses replicate is one of the most characteristic features that differentiates retroviruses from other viral families. The replication of retroviruses includes two phases: the first phase includes the entry of the virus into the cell, reverse transcription, and integration into the host genome. All the processes of the first phase utilize viral proteins. The second phase includes transcription, translation, assembly, and budding; these steps utilize host cell machinery. Briefly:

A. Attachment and Entry

The first important phase of replication is the attachment and entry of the virus into the host cell (Fig I.3). Attachment takes place through the SU portion of the virus envelope, which binds to the protein receptor found on the surface of the cell. In the case of HIV, the virus infects the cells of the immune system, which are responsible for protecting the body from the invasion by other enemies via the CD4 receptor (Clapham et al., 1999). In addition to the receptor, entry of the virus into the host cell also requires special co-receptors, the chemokine receptors. When the virus binds to the co-receptor, it activates and enhances the TM portion of the virus envelope to start the fusion process between the virus and the host cell membranes (Berger, et al., 1999). Mutations or deletions in the co-receptor genes may lead to a decrease in the permissibility to entry so that the virus may not be able to enter the cell.

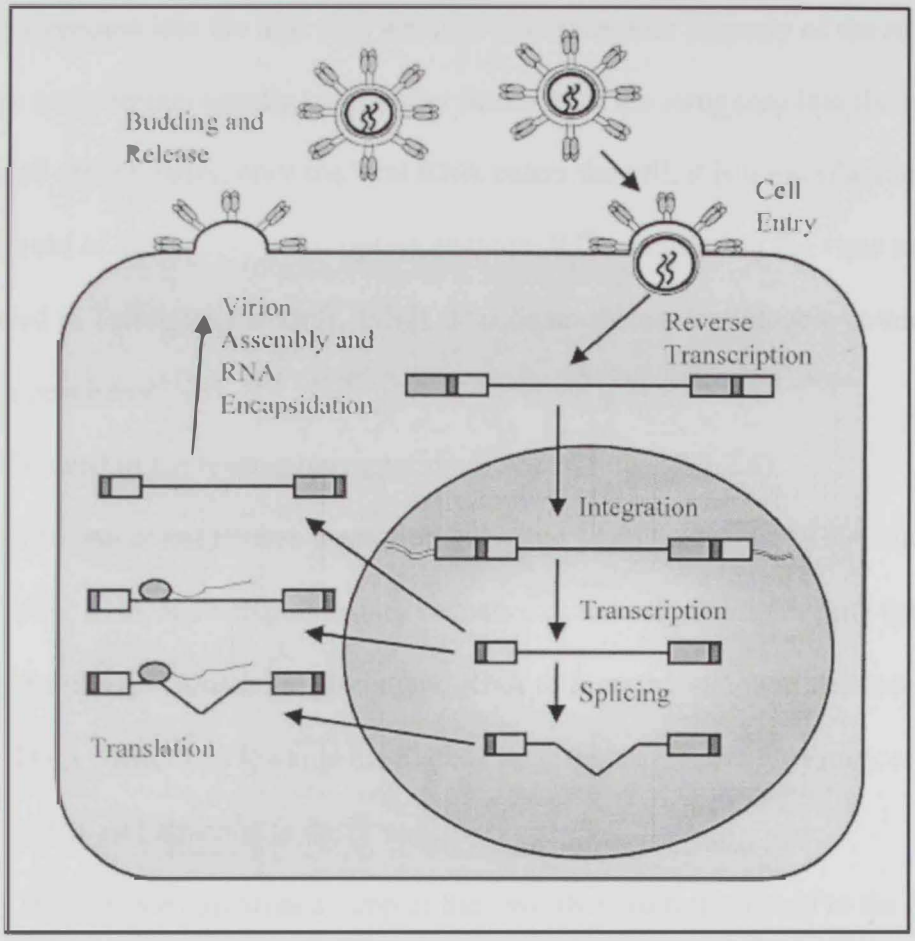

Figure I.3. Retroviral replication cycle. The replication cycle starts with the attachment of the virus to the receptor and entering the cell. Reverse transcription generates a double stranded DNA called the provirus, which is then transported to the nucleus and integrated into the chromosomal DNA. The viral RNA is then transcribed, and either spliced or leaves the nucleus unspliced. Translation and assembly takes place in the cytoplasm. The viral RNA gets packaged into the virus particles and the virus is released by budding and matures to form infectious virus.
[adapted from *Browning*, 2001]

B. Reverse Transcription

A critical part of the retroviral replication is the reverse transcription and integration of the virus genome into the host cell, which is also a peculiar property of the retroviruses. Reverse transcription usually begins after the entry of the virus core into the cytoplasm. Unlike all other viruses, once the viral RNA enters the cell, it is transcribed into DNA with the aid of the reverse transcriptase enzyme (RT) contained in the virus nucleocapsid (reviewed in Telesnitsky & Goff, 1997). This linear molecule of double-stranded DNA is called a provirus.

Steps involved in the reverse transcription of retroviruses (Fig. I.4):

1. The process of reverse transcription begins when the primer tRNA binds to the viral RNA at a complementary sequence called primer-binding site (pbs). After that the tRNA uses the plus strand RNA to form the minus strand strong stop DNA (-sssDNA) towards the 5' end and generate U5 and R sequences with the tRNA still attached to the 5' end.
2. The next step involves a jump of the –sssDNA from the 5' end to the 3' end of the genome. This jump requires the degradation of the 5' end of the RNA genome that has been replaced by the formation of ssDNA by the aid of the RNase H activity of RT. This step facilitates the binding of the ssDNA to the R sequences at the 3' end of the genome.
3. The binding of –sssDNA provides a suitable primer-template structure which helps in the process of the elongation of –sssDNA to form the long minus-strand products.

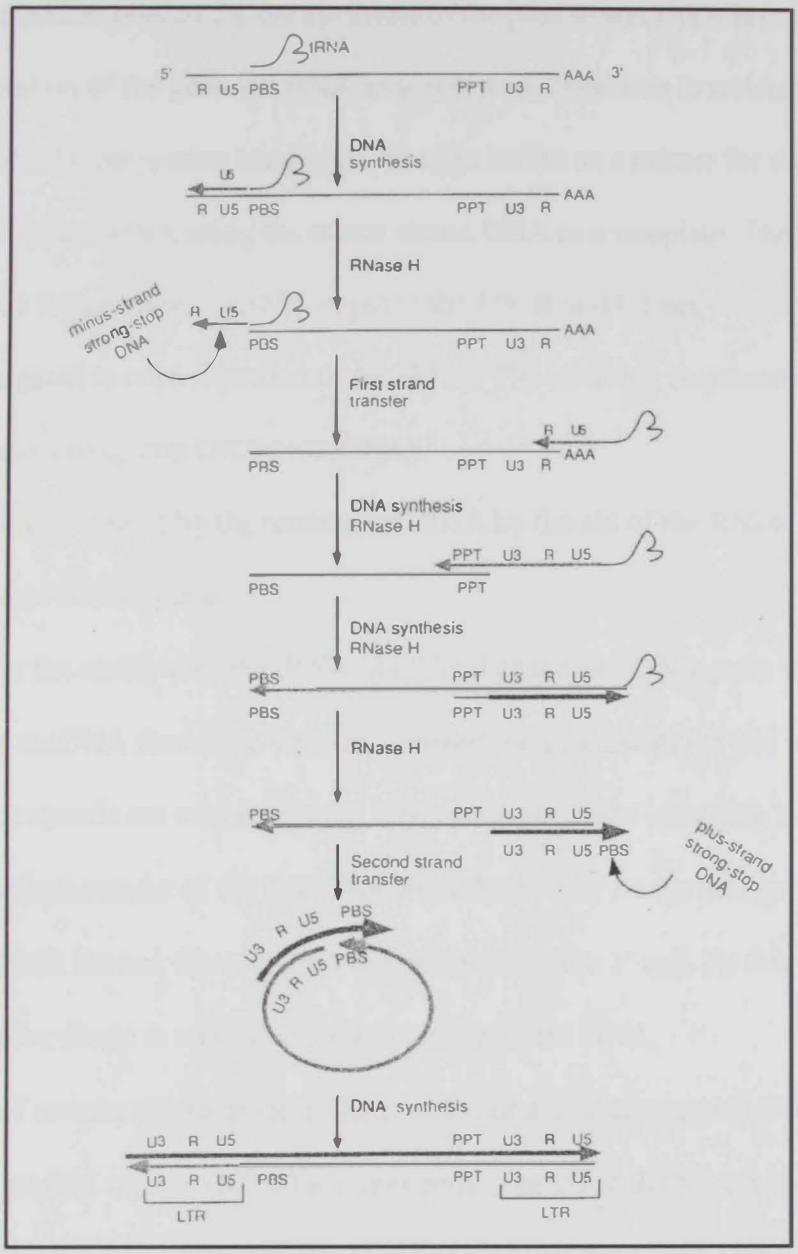

Figure 1.4. Process of reverse transcription of the retroviral genome.
[adapted from *Retroviruses*, 1997]

4. The primer needed for the synthesis of the plus strand DNA is created by the digestion of the genomic RNA in which a small portion is resistant to degradation called the polypurine tract (ppt). The ppt serves as a primer for the synthesis of plus strand DNA using the minus strand DNA as a template. The synthesis takes place towards the 5' end by copying the U3, R and U5 sequences and then elongated to copy a portion of the tRNA. The resulting sequence is called plus strand strong stop DNA (+sssDNA).
5. This is followed by the removal of tRNA by the aid of the RNase H activity of the reverse transcriptase.
6. After the removal of the tRNA, the 3'end of the +sssDNA pairs with the 3'end of the –sssDNA forming a circular intermediate that shares the pbs sequences.
7. Both strands are now elongated. The extension of the –sssDNA is coupled with the displacement of the +sssDNA from the 5' end. As the elongation of the –sssDNA occurs, the +sssDNA is transferred to the 3' end. At this point, this circular shape is opened and results into a linear DNA.

The result of reverse transcription is the creation of a double stranded DNA molecule with long terminal repeats (LTRs) at either ends. The U3 at the 5' end comes from the 3' end, while the R/U5 sequences at the 3' end come from the 5' end of the virus genome.

C. Integration

Integration is the process in which the viral DNA integrates with the host chromosomal DNA. Integration is a crucial feature in the life cycle of the retroviruses and is required for efficient replication of retroviruses. Mutant retroviruses that are unable to integrate

cannot spread the infection. A key step that must occur before integration is the entry of the viral DNA into the nucleus. This takes place due to the presence of nuclear localization signals on the viral DNA. The actual integration takes place by the aid of the integrase protein (IN), which acts to insert the linear DNA formed previously during reverse transcription into the host chromosome (reviewed in Goff, 2001 and Brown, 1997).

The integration process takes place in two steps (Fig. I.5):

1. The first step includes the removal of the two terminal nucleotides at the 3' end of the linear DNA by the IN protein that results in the formation of recessed 3' ends and protruding 5' ends.
2. During the second step, the 3'OH ends are used in a strand transfer to attack the phosphodiester bonds of the cellular DNA, making a breaks in the cellular DNA that allows the viral DNA to integrate successfully in these breaks.

In the retroviral life cycle, the integrated proviral DNA is the template used for transcription of the viral mRNAs. Therefore, formation of an integrated proviral DNA is a crucial part in the replication cycle.

D. Transcription and Translation

After the integration of the provirus inside the host cell, transcription of this proviral DNA starts to recreate the full-length viral RNA genome and spliced RNA molecules that serve as templates for the production of viral proteins. Retroviral transcription takes place by the aid of the host cell RNA polymerase II (Fig. I.6). Because there are two similar LTRs in the proviral DNA, transcription may begin at both the 5' or the 3' ends although

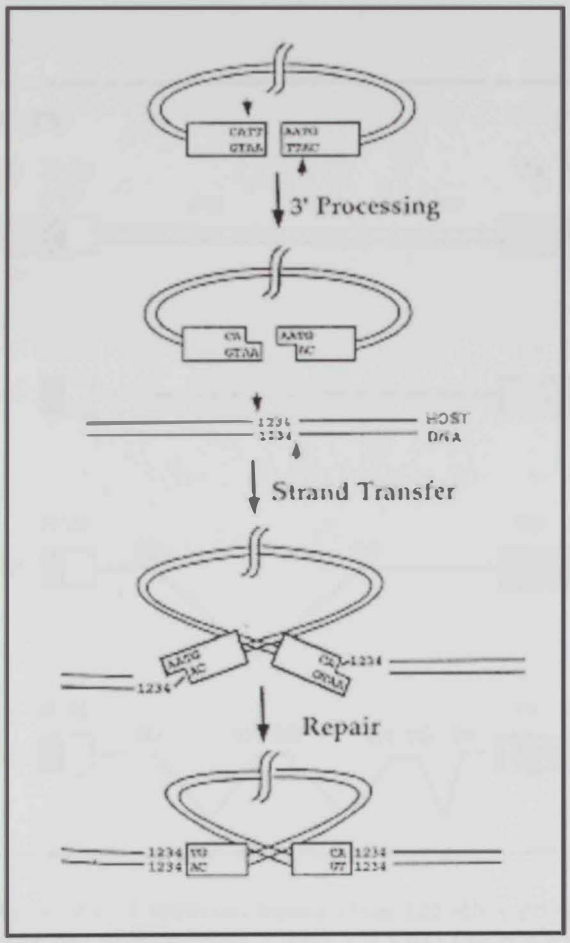

Figure I.5. Steps in the integration of the viral DNA.
[adapted from *Fundamental Virology*, 2001]

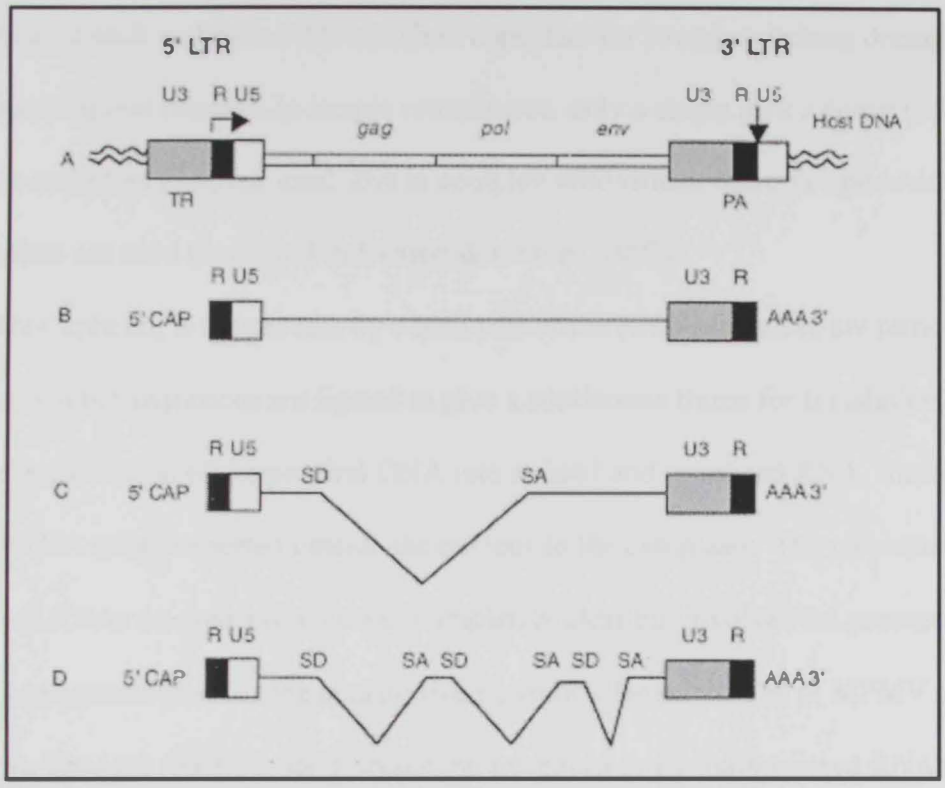

Figure 1.6. Overview of retroviral transcription and RNA processing: A typical proviral structure (A) with identical 5' and 3' LTRs. The horizontal arrow marks the start site of transcription. The vertical arrow denotes the site of 3'end processing and polyadenylation in the RNA transcript. All viruses synthesize full length genomic RNA (B). The subgenomic-sized transcripts illustrate some of the possible products of alternative splicing. In simple retroviruses (C), only a single splice donor, or, occasionally, two splice acceptors are used. In complex viruses (D), multiple spliced products are found. (TR) transcriptional control elements that function in DNA; (PA) polyadenylation and 3'–end processing signals that function in RNA; (CAP) posttranscriptional modification of 5' termini of all transcripts; (SD and SA) spliced donor and acceptor signals for splicing (C); (AAA) polyadenylation of 3' termini of all transcripts.

[adapted from *Retroviruses*, 1997]

the 5' LTR is generally more utilized than the 3' LTR. The full length RNA transcript contains a copy of all the information encoded in the proviral DNA in addition to a short direct repeat at each end called "R" which is important for strand switching during the reverse transcription process. In simple retroviruses, only a single splice donor (SD) or two splice acceptors (SA) are used. But in complex retroviruses multiple splice donors and acceptors are used (reviewed in Rabson & Graves, 1997).

RNA splicing is the process by which part of the RNA sequences are removed and the remaining sequences are ligated to give a continuous frame for translation. After complete transcription of the proviral DNA into spliced and unspliced RNA, these retroviral RNA are transported outside the nucleus to the cytoplasm. The mechanism by which these RNAs are transported is not completely clear but involves the presence of *cis*-acting sequences (such as the constitutive transport element (CTE) in MPMV or Rev Responsive Element (RRE) in lentiviruses) on unspliced or partially spliced RNAs that interact with either viral (such as Rev in HIV, SIV and FIV) or cellular transport proteins (such as CRM-1) to move these RNAs out of the nucleus and into the cytoplasm for translation and incorporation into the newly forming virus particles (reviewed in Goff, 2001, Rabson & Graves, 1997).

E. Assembly and Budding

The assembly process is initiated by the *gag*, *pro*, *pol* and *env* genes which are common to all retroviruses. The *gag* gene is translated into a polyprotein which has a major role in the assembly process. It contains the sequence needed for MA, CA and NC proteins and is translated from the unspliced viral RNA on free ribosomes in the cytoplasm. The

products of the *pro* and *pol* genes provides the enzymes- the protease (PR), reverse transcriptase (RT), and integrase (IN)- critical for virus replication and are synthesized from the same unspliced RNA as the *gag*. Spliced RNA transcripts are used to make *env* proteins via the golgi apparatus that are ultimately delivered to the membrane of the host cell for the assembly process. This is followed by the maturation of the virus particles using the viral protease to cleave the large polyprotein precursors into the mature protein subunits such as MA, CA and NC proteins that form the mature infectious virus particle. In some retroviruses, virion maturation takes place before budding (such as types B & D), while in others (such as HIV), particle maturation takes place after budding (reviewed in Swanstrom & Wills, 1997).

Brief History and Significance of FIV

Feline immunodeficiency virus (FIV) was isolated from a large multiple cat household in Petaluma, California in 1986 (Pedersen et al., 1987). This discovery was initiated by an outbreak of acquired immunodeficiency-like disease among a large group of feline-leukemia-virus-negative cats housed in the same pen (Pedersen et al., 1987). Since that time, FIV has been the target of intensive vaccine development efforts, and nowadays, it is becoming the lentivirus of choice to be used as safe vector in gene therapy.

Epidemiology

A. Host Range: All of the present isolates of FIV have come from domestic cats. However, many sera from wild Felidae, which includes African lions and cheetahs, Asian lions and tigers, South American jaguars, and North American bobcats and panthers have

shown cross-reactivity with structural antigens of FIV (Barr et al., 1989; Letcher et al., 1991; Lutz et al., 1992; Olmsted et al., 1992).

B. Geographic Distribution: FIV has been identified in many regions of the United States, Canada, Europe, South Africa, Japan, China, Australia, and New Zealand. Domestic cats spread from Europe to these countries with the early traders and explorers thousand of years ago which suggests that FIV has infected cats for a long time (reviewed in Pedersen, 1993).

General Characteristics and Morphology

FIV has a worldwide distribution among feral and domesticated cats and in many cases induces immunodeficiency disease analogous to that of human AIDS. FIV is genetically homologous to HIV in genome organization and like HIV, exhibits enormous sequence variation throughout the range of host species (reviewed in Pedersen, 1993).

The virus buds from the plasma membrane in the same way as other retroviruses. The virions are around 105-125 nm in diameter, the shape is spherical to ellipsoid, and have short, poorly defined envelope projections. The virus buds in a typical crescent-shaped appearance of C- and D-type retroviruses.

FIV has a buoyant density in sucrose of 1.15-1.17 g/cm^3. It is damaged by common virucidal concentrations of several disinfectants like chlorine, quaternary ammonium compounds, phenolic compounds, or alcohol and becomes inactivated by heating at 60°C for few minutes. It can survive for over 2 years in tissue culture media at -70°C (reviewed in Pedersen, 1993).

Cell Tropism of FIV

Unlike primate lentiviruses, FIV does not use the CD4 receptor for the virus entry but the chemokine receptors (Brown et al., 1991; Willett et al., 1997). FIV replicates in primary feline blood mononuclear cells, thymus cells, and spleen cells that have been stimulated with concanavalin A (ConA) and maintained on human recombinant interleukin-2 (IL-2) (Pedersen et al., 1987; Yamamoto, et al., 1988). The cellular tropism of FIV is restricted to feline cells. FIV will not replicate in non-feline cell lines such as primary ConA- and IL-2-stimulated canine blood mononuclear cells and lymphocytes from other species (Yamamoto et al., 1988). There is some evidence that FIV will infect some human cell lines *in vitro,* but virus replication is restricted. The restriction of the virus tropism seems to be at the virus entry level into the host cell and at the level of gene expression (Miyazawa et al., 1992). The sequence variability found in the FIV envelope genes is directly related to its host range (Hohdatsu et al., 1996; Pancino et al., 1995; Greene et al., 1993; Maki et al., 1992; Phillips et al., 1990), while the activity of FIV LTR varies with different strains of FIV and in different host cell lines (Sparger et al., 1992; Miyazawa et al., 1992 and 1994).

Gene Therapy

Genes are considered the basic functional units of heredity which are carried on chromosomes. Genes are very specific sequences of nucleotides that have codes for the formation of proteins that perform most life functions and make the majority of cellular structures. When genes are changed, the encoded proteins are unable to do their normal functions, so genetic disorders can occur. That is why gene therapy is used for treating

defective genes which are responsible for disease development (http://www.ornl.gov/sci/techresources/ Human_Genome/medicine/genetherapy.shtml).

Researchers are using different techniques for treating defective genes. Of these, a normal gene can be inserted into a non-specific place within the genome to replace a defective gene. Towards this end, a gene carrier called a "vector" is used to deliver the desired gene to the patient's target cells. Nowadays, the most common vector is a virus that has been manipulated to carry normal human DNA. Scientists have tried to take advantage of this and minimize the virus genome to remove disease-causing genes and insert therapeutic genes (http://www.ornl.gov/sci/techresources/Human_Genome/medicine/genetherapy.shtml).

A. Non-viral options for gene therapy

There are several non-viral options for gene delivery. The simplest method is the direct introduction of therapeutic DNA into target cells. The limitation is that it can be used only with certain tissues, is inefficient, and requires a very large amount of DNA. Another non-viral approach includes the creation of an artificial lipid sphere. This liposome, which has the therapeutic DNA, is capable of passing the DNA through the target cell's membrane. Researchers are also experimenting with introducing a 47th (artificial human) chromosome into target cells. This chromosome would exist independently alongside the standard 46 chromosomes--not affecting their workings or causing any mutations. It would be a large vector capable of carrying substantial amounts of genetic code, and scientists are hoping that because of its construction and autonomy, the body's immune systems would not attack it. The limitation is the difficulty in

delivering such a large molecule to the nucleus of a target cell (http://www.ornl.gov/sci/techresources/ Human_Genome/medicine/genetherapy.shtml).

B. Viral Vectors for Gene Therapy

There are several types of viruses that have been used as gene transfer vectors, including adenoviruses, adeno-associated viruses, herpes simplex virus and retroviruses. Each has its own advantages and disadvantages that have been discussed below:

Adenoviral vectors: A class of viruses with double-stranded DNA genomes that cause respiratory, intestinal, and eye infections in humans. The viruses infect with high efficiency, but don't integrate their DNA in the host cell. Adenoviruses are able to transfer genes into most tissues of the body (reviewed in Culver, 1996). These vectors have many advantages; 1) high efficiency of gene transfer regardless of the state of the tissue. In other words, they can insert the genes in both dividing and non-dividing cells. 2) They can produce virus at very high titers, and 3) these viruses can be effectively delivered using aerosol. As in all vector systems, the adenoviral vectors also have many disadvantages, including 1) Lack of tissue specificity. These vectors appear to be able to infect all cells, which could results in toxicity in normal surrounding cells. 2) Lack of ability to integrate into the host cell, thus, long term expression of these vectors is a problem. 3) *In vivo* application of adenovirus vectors results in an immune response to the viral inoculum that can limit the survival of the transduced cells.

Adeno-associated viral (AAV) vectors: AAV are a class of small, single-stranded DNA viruses that can insert their genetic material at a specific site on chromosome 19 (reviewed in Culver, 1996). AAV is considered a dependent virus because it requires co-

infection with another virus to be infectious. The advantages of AAV is its ability to transduce a variety of cell types at high efficiency; also it is not associated with any known human diseases, and it is not immunologically reactive. The limitation of this vector system is that the recombinant vectors may lose their non-random integration which may increase the possibility of insertional mutagenesis. Also, these vectors can carry only very small gene sequences, excluding the possibility of gene therapy with large genes.

Herpes simplex viral (HSV) vectors: HSV is a class of double-stranded DNA viruses that specifically infect neurons. They are lytic, non-integrating DNA viruses and have the ability to generate high viral titers and carry large genes, but their limitation is that they can reactivate latent herpes virus (reviewed in Culver, 1996).

Retroviral vectors: Retroviral vectors have advantages when compared to many other gene transfer systems (reviewed in Miller, 1997). They have the ability to infect a wide range of cell types. They integrate genetic material into the target cells, so that the host cells as well as all the subsequent progeny of the host cell maintains the therapeutic gene stably for the life of the cell. Unlike the other viral system, retroviral vectors can carry large coding capacities and importantly, the host does not elicit an immune response to the retroviral vectors. They can also express the gene of interest at high levels. One of the important advances in retroviral vector design has been the development of a retroviral packaging cell line which provides all the viral structural proteins in *trans* from independent vectors thereby preventing the formation of a replication competent virus (Mann et al., 1983; Watanabe and Temin 1983).

Retroviral vectors based on "lentiviruses" such as HIV and simian immunodeficiency virus (SIV) have the added advantage that they can also infect non-dividing cells (Miller, 1997). This is a major requirement for gene therapy in most cases since cells of the target organs of gene therapy, such as heart, liver, lung, muscles, brain, etc. divide very slowly. However, the use of primate lentiviral vectors in humans are not very promising because it raises some important safety concerns such as the generation of replication-competent virus with related endogenous viruses. Therefore, FIV is being explored as one of the possible alternatives for gene therapy. Phylogenetic comparison with other primate lentiviruses has shown that FIV is only distantly related to HIV or SIV (Bachmann, et al. 1997; Elder, et al., 1993; Olmsted, et al., 1989; Olmsted et al., 1992; Talbott et al., 1989). The use of FIV based lentiviral vectors is promising for many other reasons: 1) primates are not the natural host of FIV, 2) FIV can infect and propagate in dividing as well as non-dividing cells (Johnston et al., 1999; Poeschla et al., 1998a; Wang et al., 1999), and 3) feline models are much cheaper and easily available (Miller, et al., 2000).

RNA Encapsidation

RNA encapsidation is the process by which retroviruses package their full-length, unspliced genomic RNA preferentially over spliced viral and cellular RNAs into the virus particles (reviewed in Berkowitz et al., 1996 and Swanstrom and Wills, 1997). The specificity of packaging has been attributed to the interaction between a *cis*-acting sequence, the packaging signal (E or Ψ) on the retroviral RNAs, and the *trans*-acting nucleocapsid region of the Gag polyprotein. The nucleocapsid region of the Gag

polyprotein provides zinc-binding domains that are critical elements for the interaction with the packaging signal present in the retroviral genomic RNA (Dorfman et al., 1993; Gorelick et al., 1993; Sakaguchi et al., 1993; Zhang and Barklis, 1995). The packaging signals, in turn, are thought to interact with the nucleocapsid protein at a structural level, and as such have been shown to fold into complex stem loop structures (Berkowitz et al., 1996). This may partially explain cross-packaging between retroviruses that lack any apparent sequence similarity between each other, such as murine leukemia and spleen necrosis viruses, MLV/SNV (Certo et al., 1998) or HIV/FIV (Browning et al., 2001).

The primary RNA packaging determinants (the major packaging signal) lie at the 5' end of the retroviral genome; however, the exact sequence requirement differs between different retroviruses. For some retroviruses, such as SNV, Rous sarcoma virus (RSV), and feline leukemia virus (FeLV), the major packaging signal lies within the 5' UTR between the pbs and the start of *gag* (reviewed in Linial and Miller, 1990); while in others, the packaging signal seems to extend into the 5' end of the *gag* gene, such as for MLV (Bender et al., 1987), bovine leukemia viruses (BLV) (Mansky et al., 1995), MPMV (Schmidt et al., 2003), and HIV-1 (Parolin et al., 1994). However, other regions of the viral genome have also been implicated as containing minor packaging determinants, such as the 3' UTR (Sorge et al., 1983; Ashcoff et al., 1999; Yu et al., 2000) and the LTR (Murphy and Goff, 1989; Clever et al., 1999; Helga-Maria et al., 1999; Guan et al., 2001a). In general, the simple retroviruses are thought to contain more discrete packaging determinants localized to small segments of the 5' end of the viral genome, while those of the complex retroviruses are thought to be more spread out (Berkowitz et al., 1996; Banks et al., 1997). The only exception to the rule are the

spumaviruses. There is some data that suggests that a central region in *pol* may be involved in RNA packaging (Heinkelein et al., 1998; Erlwein et al., 1998).

With an increase in studies on retroviral packaging determinants, the emerging picture suggests that the location of the primary packaging determinant in relation to the major splice donor (mSD) may be more instructive as to how different retroviruses have developed strategies to differentiate between full length and spliced RNAs. For example, the core packaging signals of the γ retroviruses SNV, MLV, and FeLV are located downstream of the mSD in the viral intron, thus allowing for packaging only of the unspliced viral RNA containing the Ψ over spliced mRNAs which lacks it (Watanabe and Temin, 1982; Adam and Miller, 1988; Burns et al., 1996). Furthermore, in MLV, the genomic RNAs are sorted into two pools---one destined for translation, while the other reserved for packaging (Dorman and Lever, 2000). However, in the α retroviruses such as ALSV, the packaging signal is located upstream of the major SD, allowing for both spliced and unspliced mRNAs to be packaged into the viral genome (Banks et al., 1998). Since *env* mRNA in these viruses is the only spliced species produced, sequestration of this mRNA spatially in the rough endoplasmic reticulum may keep it away from the Gag polyprotein which is expressed from the free ribosomes in the cytoplasm, thus allowing specific packaging of the full-length RNA which is expressed in the cytoplasm (Banks et al., 1999).

Similarly, the core packaging determinant in the β retrovirus, MPMV, lies upstream of the mSD (Guesdon et al., 2001; Schmidt et al., 2003); however, sequences within the 5' end of *gag* seem to be equally important for packaging (Schmidt et al., 2003). Since regions within *gag* are part of the intron in MPMV, the presence of

packaging determinants within the 5' UTR upstream of the SD but extending into *gag* may help MPMV differentiate between spliced and unspliced mRNAs. Using biochemical and phylogenetic analyses, and computer modeling, the first 275 bp of the MPMV 5' UTR and *gag* have been predicted to fold into a complex secondary RNA structure containing eight stem loops (Harrison et al., 1995) (Fig. I.7, panel A). Mutations within the first four stem loops present within the 5' UTR have revealed that only the sequences within the first stem loop and not the loop structure itself are critical for MPMV RNA encapsidation (Mustafa et al., 2004), while the role of structures within *gag* towards packaging still needs to be determined.

A somewhat different picture is observed with the δ retroviruses, the BLV/HTLV group of viruses that are similar to the spumaviruses in that the major splice donor resides within the R region of the LTR. However, the similarity ends here. Using deletion analysis of the BLV 5' region, these studies have mapped the packaging signal of BLV to two discrete discontinuous regions downstream of the mSD that fold into stem loop structures (Mansky et al., 1995; Mansky and Wisniewski, 1998). The first region comprises of the entire 5' UTR downstream of the pbs and ends within 70 bp of the *gag* region in the matrix domain, while the second region exists more downstream of the *gag* gene in the capsid domain. Region 1 folds into two stem loops that exist within the *gag* sequences only, while Region 2 folds into a single stem loop structure. All three stem loop structures seem important for packaging, and interestingly, replacement of the sequences containing the two regions with homologous regions from either HTLV-1 or HTLV-2 results in substantial packaging, suggesting that the packaging signals of the three viruses function at a structural level. Furthermore, this region allows specific

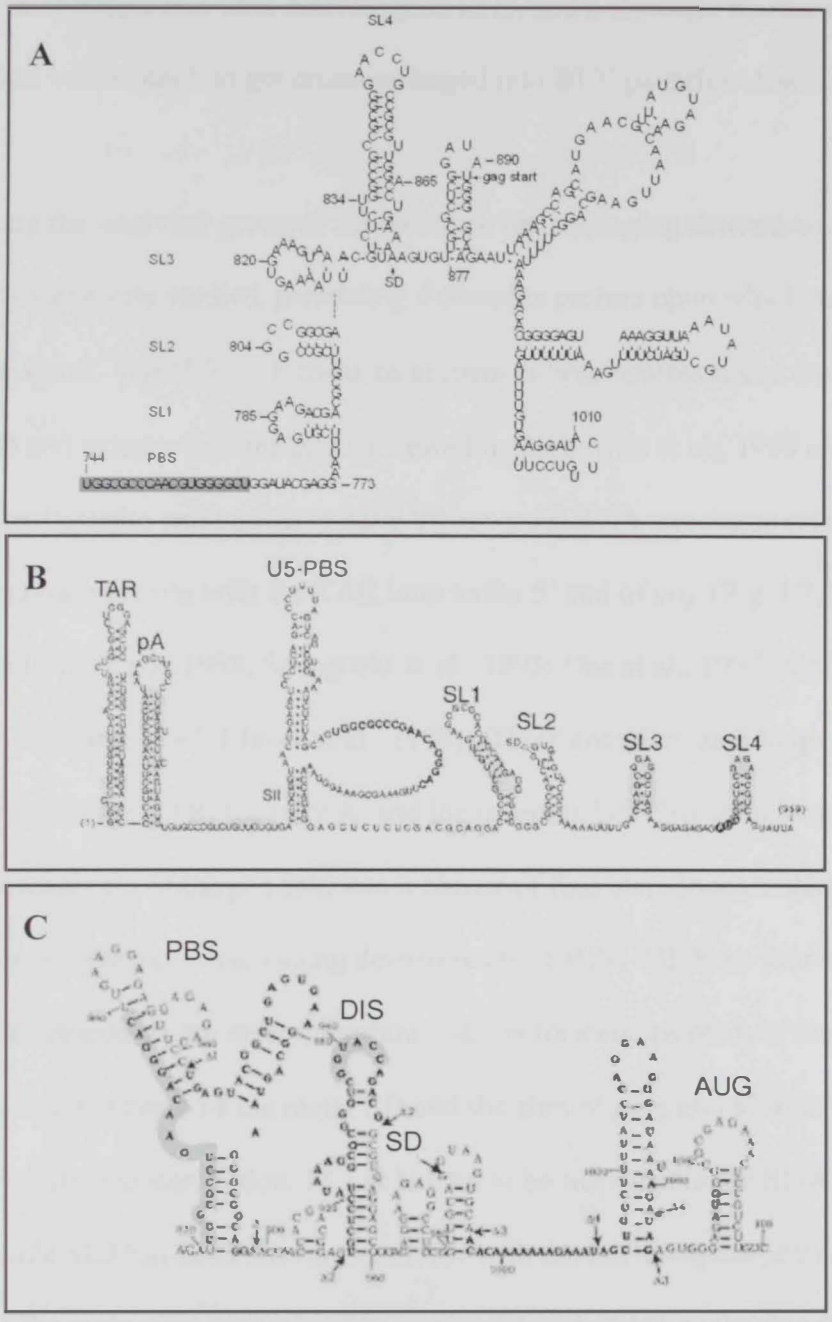

Figure I.7. The predicted secondary RNA structure of the 5' end of A) MPMV [adapted from Harrison et al., 1995], B) HIV [adapted from Clever et al., 2002], and C) SIV [adapted from Strappe et al., 2003] genomic RNAs. The major *cis*-acting sequences of the virus are highlighted by the gray shading such as the primer binding site (PBS), dimer intitiation site (DIS), the *gag* initiation codon (AUG), splice donor (SD), polyadenylation (pA), and transactivating region (TAR).

incorporation of both a non-viral heterologous RNA and a chimeric murine leukemia virus retroviral vector RNA to get cross-packaged into BLV particles (Jewel and Mansky, 2005).

Among the lentiviral group of retroviruses, the packaging determinants of HIV-1 are the most extensively studied, presenting a complex picture upon which, most investigators agree. The HIV-1 Ψ contains sequences both upstream and downstream of the major SD and extends into the *gag* (reviewed in Berkowitz et al., 1996 and Lever, 2000). It is multipartite and known to have higher order RNA structures extending from the transcriptional start site with the TAR loop to the 5' end of *gag* (Fig. 1.7, panel B) (Harrison et al., 1992 and 1998; Sakaguchi et al., 1993; Das et al., 1997; McBride and Panganiban, 1996 and 1997; Clever et al., 1999). Three complex stem loops are present within the 5' LTR (the TAR, the poly A, and the complex U5/PBS stem loops), while the sequences downstream of the pbs fold into a cluster of four stem loops called SL1-4 thought to contain the major packaging determinants of HIV. Of these four stem loops, SL1 and 3 are considered the most important. SL1 is located upstream to the major SD, SL3 is located downstream of the major SD and the start of *gag*, and SL4, is located just downstream of the *gag* start codon. SL1 is known to be responsible for RNA dimer formation, while SL3 has been shown to interact with the nucleocapsid protein. SL3 appears to be the main stem loop which is consistently related to packaging (Lever, 2000), while sequences contained within SL1, may be SL4, and other sequences present in the viral genome may play a role in maintaining the larger secondary RNA structure that forms the complex HIV-1 packaging signal (McBride and Panganiban, 1997; Lever, 2000). However, SL1-4 are not sufficient in themselves *in vivo* for packaging and other

sequences upstream to SL1 are also required (Helga-Maria, et al., 1999; Kaye, et al. 1995; McBride, et al., 1997; Parolin, et al., 1994). For example, mutation in the stem of the TAR element affect packaging significantly (Helga-Maria et al., 1999; Clever et al., 1999), while sequences within the U5-PBS between the poly A stem loop and SL1 contribute to HIV-1 RNA packaging (McBride et al., 1997, Clever et al., 1999). Additionally, the GU-rich sequences found in the lower stem of poly A stem loop and the U5-PBS complex show contribution to both dimerization and packaging (Russell et al., 2002). Since the major packaging determinant of HIV-1 resides within SL3 (found downstream of the mSD), its presence on full-length genomic RNA ensures specific incorporation into the virions over and above the spliced mRNAs.

The packaging signal of HIV-2 is less well-studied and controversial, probably due to the presence of a functional intron in the HIV-2 LTR (reviewed in Lever, 2000 and Griffin et al., 2001). Although both HIV-1 and 2 cause AIDS and maintain similar genomic organization, the two viruses have limited sequence homology, reflecting their distinct evolutionary origins (Franchini et al., 1987). Like HIV-1, the sequences involved in HIV-2 packaging are spread out and function in a context-dependent manner. Although sequences both upstream and downstream of the major SD have been implicated in the packaging of the HIV-2 genomic RNA (Garzino Demo et al., 1995; McCann and Lever, 1997; Kaye and Lever, 1999; Poeschla et al., 1998b), the HIV-2 packaging signals belong to the ALSV/MPMV category where the core packaging determinants are located upstream of the major SD (Kaye and Lever, 1998 and 1999; McCann and Lever, 1997). This conclusion is strengthened by the observation that HIV-

1 proteins can cross-package not only full length HIV-2 mRNAs non-reciprocally, but also spliced HIV-2 mRNAs (Kaye and Lever, 1998).

Since all HIV-2 mRNAs maintain the core packaging determinant, it has evolved a unique strategy to preferentially incorporate only genomic and not spliced mRNAs into the nascent virus particles. The strategy requires that the genomic RNA act as the template for the translation of the Gag polyprotein that would capture the co-translated RNA in "*cis*" for incorporation into the virus particle (Kaye and Lever, 1999). Named "*cis*-acting encapsidation", this strategy may also partially explain why HIV-2 is unable to encapsidate either HIV-1 or HIV-2 vector RNAs in *trans*.

Finally, the packaging determinants of SIV RNA have started to emerge, but the findings are controversial. SIV is more closely related to HIV-2 than HIV-1, especially in the non-coding regions of their genome (Patel et al., 2003). Like HIV-2, SIV also contains a functional intron within its LTR (Viglianti et al., 1990; Unger et al., 1991). The role of sequences within the 5' UTR towards packaging are still not clear with Patel et al., (2003) suggesting that significant packaging determinants are present both upstream and downstream of the major SD, while others showing that, similar to HIV-2, the core packaging signal of SIV resides upstream of the major SD, perhaps in the U5 stem and the dimerization initiation sequences (DIS) (Fig. I.7, panel C) (Strappe et al., 2003; Guan et al., 2000 and 2001b; Rizvi and Panganiban, 1993). The location of the packaging signal relative to the 5' methyl cap is ~470 nts (very similar in SIV and HIV-2, but much shorter in HIV-1) Strappe et al., further hypothesize that this distance may be important for packaging, since it may affect the competition between translation and encapsidation that the genomic RNAs must undergo. The important conclusion from

their observations is that for retroviruses, it is not the position of the SD relative to the core packaging determinant that dictates the strategy that the virus may use for encapsidation, rather the distance between the cap site and the core packaging determinants. These observations are being further investigated experimentally.

Since incorporation of the genomic RNA into the budding virion is one of the last steps in the retroviral life cycle, successful packaging of the genomic RNA is dependent upon a host of steps that should have taken place successfully (Fig. 1.8). These steps include binding of the virus to the cell surface, fusion of the two membranes and entry of the viral core into the cytoplasm. This is followed by successful reverse transcription of the incoming genomic RNA into cDNA, transport of the cDNA to the nucleus, its integration into the host genome, its subsequent transcription into mRNAs, and finally successful transport of the full-length genomic RNA from the nucleus to the cytoplasm into the correct cellular compartment where assembly takes place. The emerging paradigm of studying RNA packaging dictates that when understanding the effect of the introduced mutations, one must keep in mind that an alteration of any one of the steps outlined above could negatively affect RNA packaging, without affecting the packaging process directly (Lever, 2000).

Among lentiviruses, our interest has been to define the packaging signal of FIV, a lentivirus of non-primate origin. Not much work has been done to understand how lentiviruses of non-primate species package their RNA genomes. We are also interested in FIV RNA packaging since FIV-based gene transfer vectors are being considered as a

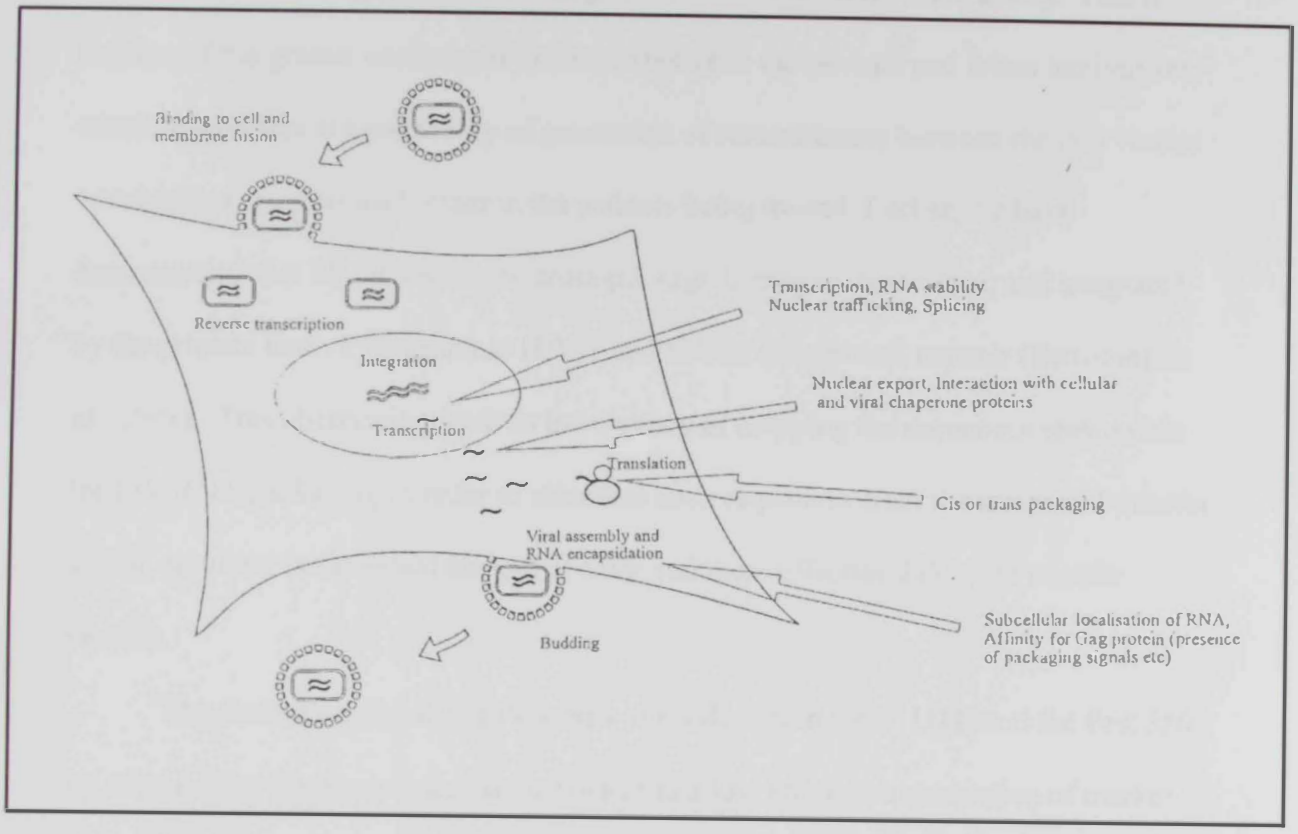

Figure I.8. The packaging pathway. (For details, see text).

[adapted from Lever, 2000]

potentially practical vector system for human gene therapy (Naldini et al., 1996; Johnston et al., 1999; Poeschla et al., 1998a;Wang et al., 1999; Browning et al., 2001). This is because of the greater evolutionary distance between the primate and feline lentiviruses which may reduce the possibility of generation of recombinants between the two viruses which may encounter each other in the patients being treated. Earlier, we have demonstrated that FIV RNA can be cross-packaged, reverse transcribed, and integrated by the primate lentiviruses such as HIV-1 and SIV in a reciprocal manner (Browning et al., 2001). This observation leads to the urgency of mapping the sequences responsible for FIV RNA packaging in order to eliminate such sequences from the integrated transfer vector, resulting in the establishment of safer and more effective FIV gene transfer vectors.

The packaging signal of FIV seems to reside within the 5' UTR and the first 350 bp of *gag* since these sequences are sufficient to allow efficient transduction of marker genes carried by FIV-based vectors (Browning et al., 2001, Johnston et al., 1999; Poeschla et al., 1998a). These studies were followed by a more systematic deletion analysis of the FIV 5' UTR and *gag* sequences in our laboratory using subgenomic constructs and slot blot analyses on virion and cellular RNAs. These studies revealed that like HIV-1, the FIV packaging determinants were complex and multipartite consisting of two discontinuous core regions, one located in the 5' UTR upstream of the major SD from R/U5 to the first 120 bp of UTR, while the other found in the first 100 bp of the *gag* gene (Browning et al., 2003 a & b). These two core elements were equally important and simultaneously required for packaging since deletion of one of these elements reduced packaging to a similar 4-6 fold level, while deletion of both elements together reduced

packaging to a drastic 40- fold lower level, but not completely (Browning et al., 2003 a & b).

Another group studying FIV RNA packaging confirmed some of these observations, while raising many questions as well (Kemler et al., 2002 & 2004). Using RNase protection assays on virion and cellular RNAs of full length genomic and subgenomic constructs, these studies showed that indeed the first 90 bp of *gag* were critical for FIV RNA packaging, while the region downstream of the mSD and beginning of *gag* was dispensable, in agreement with our observations. However, they suggested that the major FIV packaging determinants were constrained to the R/U5 region with the first 154 bp of the 5'UTR also playing some role. Additionally, they found no role of 875 bp of *env* or the 3' LTR in FIV RNA packaging.

Objectives

This project was undertaken to get a clearer picture of the packaging determinants of FIV. We started with observations that FIV packaging determinants seemed to reside within two discrete regions (Browning et al., 2003 a & b) and asked whether the sequences in between the two core packaging determinants were important for packaging. Since other studies came out during the course of our investigations (Kemler et al., 2002 & 2004), we further asked what the role of the FIV LTR sequences was towards packaging and which sequences constituted the core packaging determinants.

To answer these questions, two independent approaches were taken to fine map FIV RNA packaging determinants using both genetic and structural approaches:

1) An incremental deletion analysis was conducted of the region in between the two core regions in subgenomic constructs. In a separate series, the deleted region was substituted simultaneously with heterologous sequences of the same length to determine whether the spacing between the two core regions was important or the actual sequences themselves (Chapter III).
2) The R/U5 region at the 5' end and the 3' LTR at the end of the FIV genome were independently tested in subgenomic and heterologous vectors for their effects on FIV RNA packaging (Chapter IV).

These vectors were tested in an *in vivo* packaging assay using semi-quantitative RT-PCR analysis of virion and cytoplasmic RNAs. Test of these constructs revealed that, as suspected earlier, FIV packaging determinants are complex and multipartite. More specifically, we found that:

1) The first ~ 150 bp of the 5' UTR and ~ first 100 bp of *gag* constitute the core packaging determinants (Chapters III & IV).
2) The region in between the two core packaging determinants is neither required for vector RNA packaging nor propagation of the FIV transfer vector RNA (Chapter III).
3) FIV R/U5 contains minor packaging determinants that increase the packaging of FIV transfer vector RNAs by 2-3 fold (Chapter IV).
4) Contrary to published findings, the FIV 3' LTR and in particular the U3 region harbors packaging determinants of minor nature (Chapter IV).

Finally, using computer predictions of sequences found to be important for FIV RNA packaging in our functional assays, we identified a specific structural element within the 5' end of the FIV genome that may have a critical role in FIV RNA packaging.

Chapter II

MATERIALS & METHODS

Genome Numbering System

Nucleotide designation for FIV Petaluma (34TF10) strain is based on GenBank accession number M25381 (Talbott, *et al,* 1989).

Plasmid Construction

FIV packaging construct. The packaging construct, MB22 which provides the viral structural proteins, has been described previously (Browning et al., 2001) (Fig. II.1). In brief, it uses the human cytomegalovirus (hCMV) as promoter to express all the genes of FIV except *env* and *orf2,* and uses the bovine growth hormone (BGH) poly A sequences to stop transcription.

Envelope expression construct. The vesicular stomatitis virus (VSV) envelope protein G expression construct (VSV-G), MD.G, was provided by Dr. Didier Trono (Salk Institute, La Jolla, CA) and has been described previously (Naldini, *et al*, 1996).

FIV control transfer vector. The control vector, MB15, has been described previously (Browning et al., 2001) (Fig. II.1). In brief, it contains the entire 270 bp of 5' untranslated region (UTR), 100 bp of *gag* and all the necessary *cis*-acting sequences needed for packaging, reverse transcription and integration.

Transfer vectors with deletions between the two core regions. AG002 through AG004 are transfer vectors containing deletions in the 5' UTR. Of the 270 bp 5' UTR, we maintained 90, 120 and 150 bp from the 5' end of the 5' UTR while deleting the 180, 150, 120 bp upstream of the *gag* gene, respectively. These vectors were generated

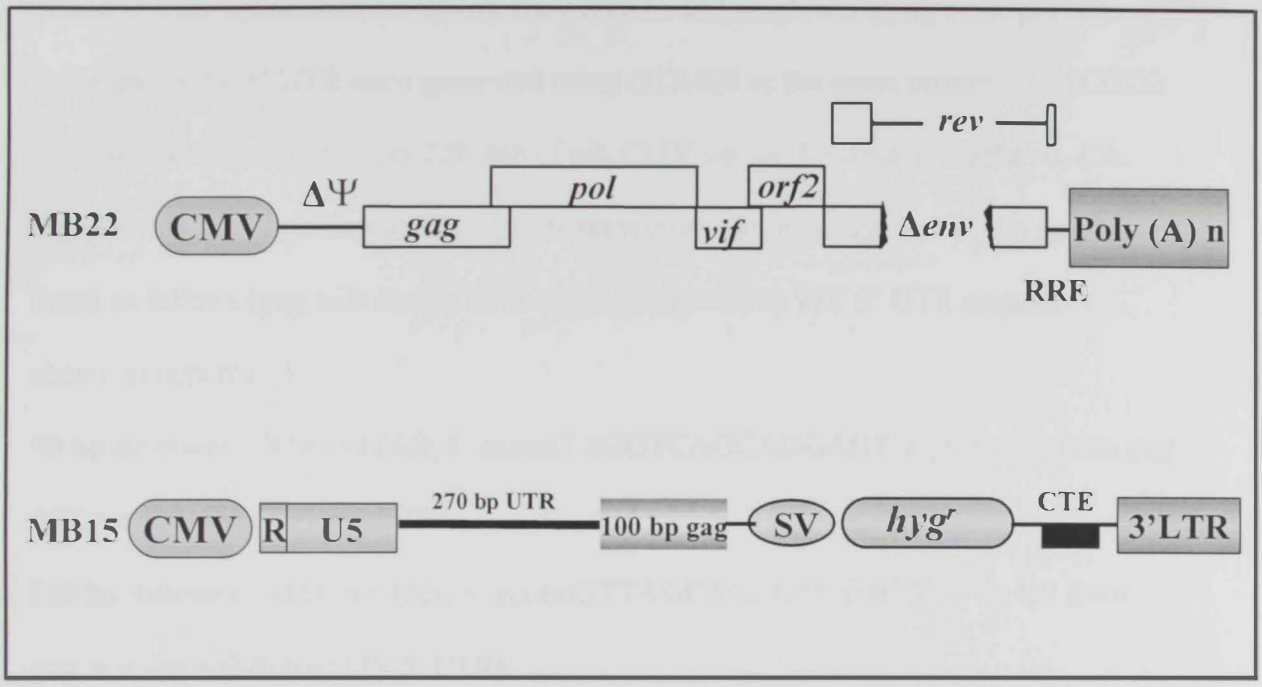

Figure II.1. Schematic representation of FIV packaging construct MB22 and FIV control transfer vector MB15. CMV, human cytomegalovirus promoter-enhancer; SV, simian virus 40 early promoter; *hygr*, *hygromycin B phosphotransferase* gene; CTE, constitutive transport element of MPMV; Ψ packaging signal.

through splice overlap extension (SOE) PCR (Gibbs et al., 1994) using TR394 as a template. To generate these vectors, two individual PCRs were performed using hybrid primers which resulted in two overlapping PCR products (Fig. II.2). These two primary products were annealed at complementary regions and amplified using outer primers. Deletions in the 5' UTR were generated using OTR405 as the sense primer (S; 5' CGCG TTGACATTG ATTAT 3'; nt 229-246 of pRcCMV vector, Invitrogen, Carlsbad, CA, representing the beginning of CMV promoter region) and hybrid primers with *gag* tails listed as follows (*gag* tails shown in lowercase followed by FIV 5' UTR sequences shown in uppercase):

90 bp deletion: OTR568 (AS; 5' ccccatTAGGTCAGCAGGAGT 3'; 6 bp tail from *gag* ATG/nt 432-446 of FIV 5' UTR).

120 bp deletion: OTR569 (AS; 5' ccccatGTTAGCAGCGTCTGC 3'; 6 bp tail from *gag* ATG/nt 462-476 of FIV 5' UTR).

150 bp deletion: OTR570 (AS; 5' ccccatCTCGAGTCCGCTTCA 3'; 6 bp tail from *Gag* ATG/nt 492-505 of FIV 5' UTR).

The 100 bp of *gag* were generated using TR394 as a template and OTR503 as the antisense primer (AS; 5' aaaaagcggccgcTCCCTTCTCCAAATTTTTTACTCTTCC 3'; FIV *gag* nt 727-701, sequences in lowercase represent artificial sites created for cloning purposes while FIV sequences are shown in upper case) and hybrid sense primers containing 5' UTR tails corresponding to the deletions listed as follows (5' UTR tails shown in lowercase followed by *gag* sequences shown in uppercase):

90 bp deletion: OTR571 (S; 5' gacctaATGGGGAATGGACAG 3'; 6 bp tail of 5' UTR corresponding to the 90 bp deletion /nt 628-642 in *gag*).

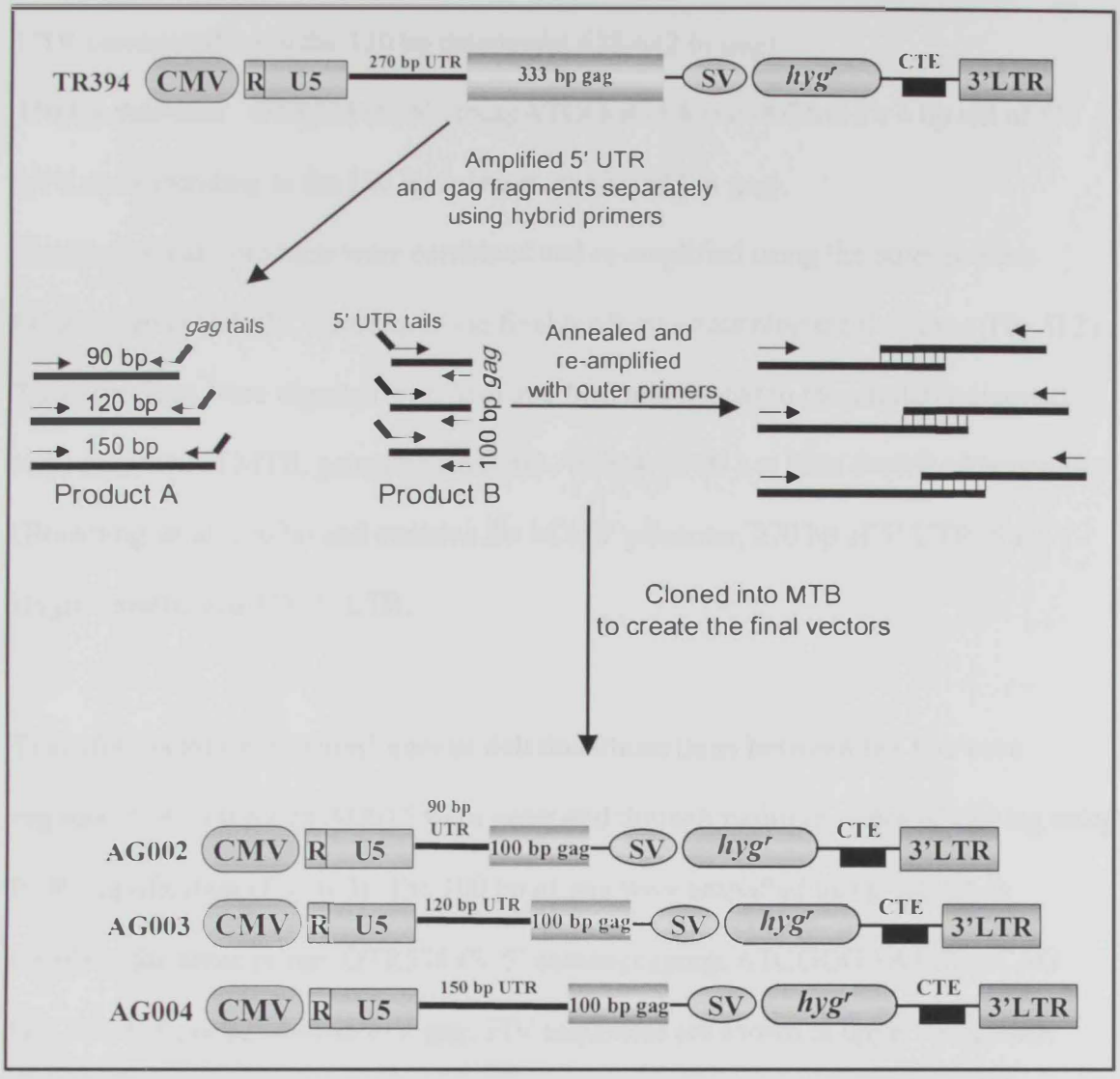

Figure II.2. Schematic representation of transfer vectors containing deletions between the two core regions. The 5' UTR and *gag* fragments were amplified separately using hybrid primers. The UTR primers contained *gag* tails and the *gag* primers contained UTR tails, resulting in products A & B. These two products were annealed and re-amplified using outer primers, resulting in a PCR product containing the required deletions. These products were digested and ligated back to create the final vectors, AG002-AG004. CMV, human cytomegalovirus promoter-enhancer; SV, simian virus 40 early promoter; *hygr*, *hygromycin B phosphotransferase* gene; CTE, constitutive transport element of MPMV.

120 bp deletion: OTR572 (S; 5' gctaacATGGGGAATGGACAG 3'; 6 bp tail of 5' UTR corresponding to the 120 bp deletion/nt 628-642 in *gag*).

150 bp deletion: OTR573 (S; 5' ctcgagATGGGGAATGGACAG 3'; 6 bp tail of 5' UTR corresponding to the 150 bp deletion /nt 628-642 in *gag*).

The two primary products were combined and re-amplified using the outer primers OTR405 and OTR503, resulting in the final products containing the deletions (Fig. II.2). These products were digested with *Spe*I and *Not*I and ligated to the similarly digested large fragment of MTB, generating AG002-AG004. MTB has been described previously (Browning, et al, 2003a) and contains the hCMV promoter, 270 bp of 5' UTR, the SV-Hygro cassette and FIV 3' LTR.

Transfer vectors with simultaneous deletions/insertions between the two core regions. AG013 through AG015 were generated through multiple stages of cloning using PCR amplification (Fig. II.3). The 100 bp of *gag* were amplified using TR394 as template, the sense primer OTR574 (S; 5' aaaaaagcggccgcATGGGGAATGGACAG GGGCGA 3'; nt 628-648 in FIV *gag*, FIV sequences are shown in upper case, while sequences in lowercase represent artificial sites created for cloning purpose) and the antisense primer OTR575 (AS; 5' ccccccatcgatTCC CTTCTCCAAATTTTTTACTCT 3'; nt 727-701 in FIV *gag*). The PCR products were digested with *Not*I and *Cla*I and ligated to the similarly digested large fragment of MB23, a vector similar to MTB but without the SV-Hygro cassette, resulting in AG006. The fragments of the 5' UTR were amplified using TR394 as template and the sense primer OTR405. The deletions in the 5' UTR were generated using the antisense primers listed as follows (FIV sequences are

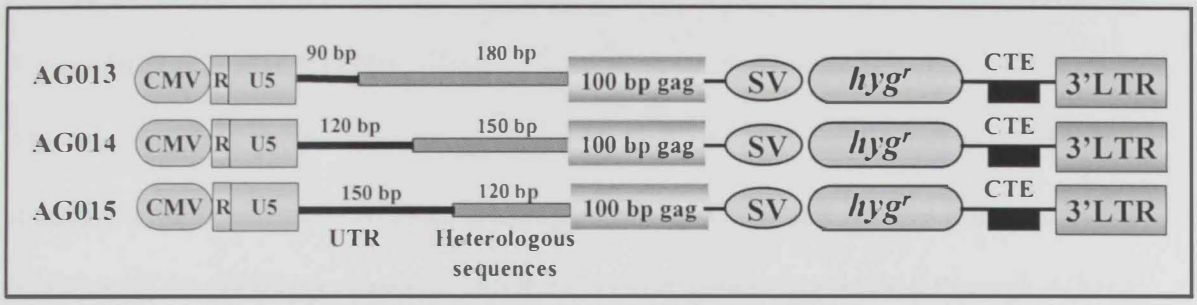

Figure II.3. Schematic representation of transfer vectors with simultaneous deletions/insertions between the two core regions (AG013-AG015). These transfer vectors maintained 90, 120 or 150 bp of 5' UTR with the addition of 180, 150, 120 bp of heterologous sequences in the presence of 100 bp of *gag*. CMV, human cytomegalovirus promoter-enhancer; SV, simian virus 40 early promoter; *hyg*^{r}, *hygromycin B phosphotransferase* gene; CTE, constitutive transport element of MPMV.

shown in upper case while sequences in lowercase represent artificial sites created for cloning purpose):

90 bp deletion: OTR576 (AS; 5' aaaaaagcggccgcTAGGTCAGCAGGAGTTCTGCT 3'; nt 426-446 in FIV 5' UTR).

120 bp deletion: OTR577 (AS; 5' aaaaaagcggccgcGTTAGCAGCGTCTGCTACTGC 3'; nt 456-476 in FIV 5' UTR).

150 bp deletion: OTR584 (AS; 5' aaaaaagcggccgcCTCGAGTCCGCTTCACTAGAG 3'; nt 486-506 in FIV 5' UTR).

The resulting PCR products were digested with *Spe*I and *Not*I and ligated to the similarly digested large fragment of AG006, creating AG007, AG008 and AG009 followed by the insertion of the SV-Hygro cassette into the *Cla*I site, generating AG010 through AG012. The heterologous sequences were generated by PCR amplification using pcDNA3 as template and the antisense primer OTR480 (AS; 5' aaaaaagcggccgcgaattcCAAGTTTA CTCATATATACTTTAGATTGAT 3'; pcDNA3 nt 4440-4411 on Invitrogen's map) and the sense primers listed below (sequences in lowercase represent artificial sites created for cloning purpose, while pcDNA3 sequences are shown in upper case):

120 bp insertion: OTR583 (S; 5' aaaaaagcggccgcggatccGAACGAAAACTCAC GTTAAGGGAT 3'; nt 4321-4344 of pcDNA3).

150 bp insertion: OTR578 (S; 5' aaaaaagcggccgcggatccTGATCTTTTCTACG GGGTCTGACG 3'; nt 4290-4313 of pCDNA3).

180 bp insertion: OTR579 (S; 5' aaaaaagcggccgcggatccCAGAAAAAAAGGAT CTCAAGAAGA 3'; nt 4201-4284 of pCDNA3).

The PCR products were digested with *Not*I and ligated to the similarly digested large fragment of AG010-AG012, resulting in the final clones AG013 through AG015.

Transfer vectors testing the R/U5 region. To study the effect of the R/U5 region independently on packaging, various constructs were made, either with or without the R/U5 region, in both the heterologous and homologous contexts (Fig. II.4).

R/U5 in the heterologous context. AG029 is transfer vector containing the R/U5 region only in pcDNA3 as a backbone. The R/U5 region was amplified using pCMV/RU5 as a template (Mustafa, et al., 2005), OTR399 as the sense primer (S; 5' cccaagcttgagctcTGTGAAACTTCGAG GAGTCTC 3'; FIV 5' LTR nt 203-223, U3/R junction), (lowercase denotes artificial sites created for cloning purpose, while FIV sequences are shown in upper case) and OTR459 as the antisense primer (AS; 5' aaaaaagcggccgcACTGCGAAGTTCTCGG CCCGG 3'; FIV nt 357-337, end of U5). The resulting PCR product was digested with *Hind*III and *Not*I, ligated to the similarly digested large fragment of pcDNA3, followed by insertion of the SV-Hygro cassette at the *Xba*I site, generating AG029.

R/U5 + 90, 120, or 150 bp of 5' UTR + 100 bp gag in the heterologous context. These vectors were made through multiple stages of cloning by modifying pcDNA3 where the region between *Nde*I site (nt 485) and the artificially created *Cla*I site (nt 951) was removed and replaced by the region between the *Nde*I site and the *Cla*I site of AG007 through AG009 (consisting of R/U5 + 90, 120, or 150 bp of FIV 5' UTR + 100 bp *gag*, respectively). This was followed by insertion of the SV-hygro cassette at the *Xba*I site (nt 984) of pcDNA3, generating AG026, AG027 and AG028.

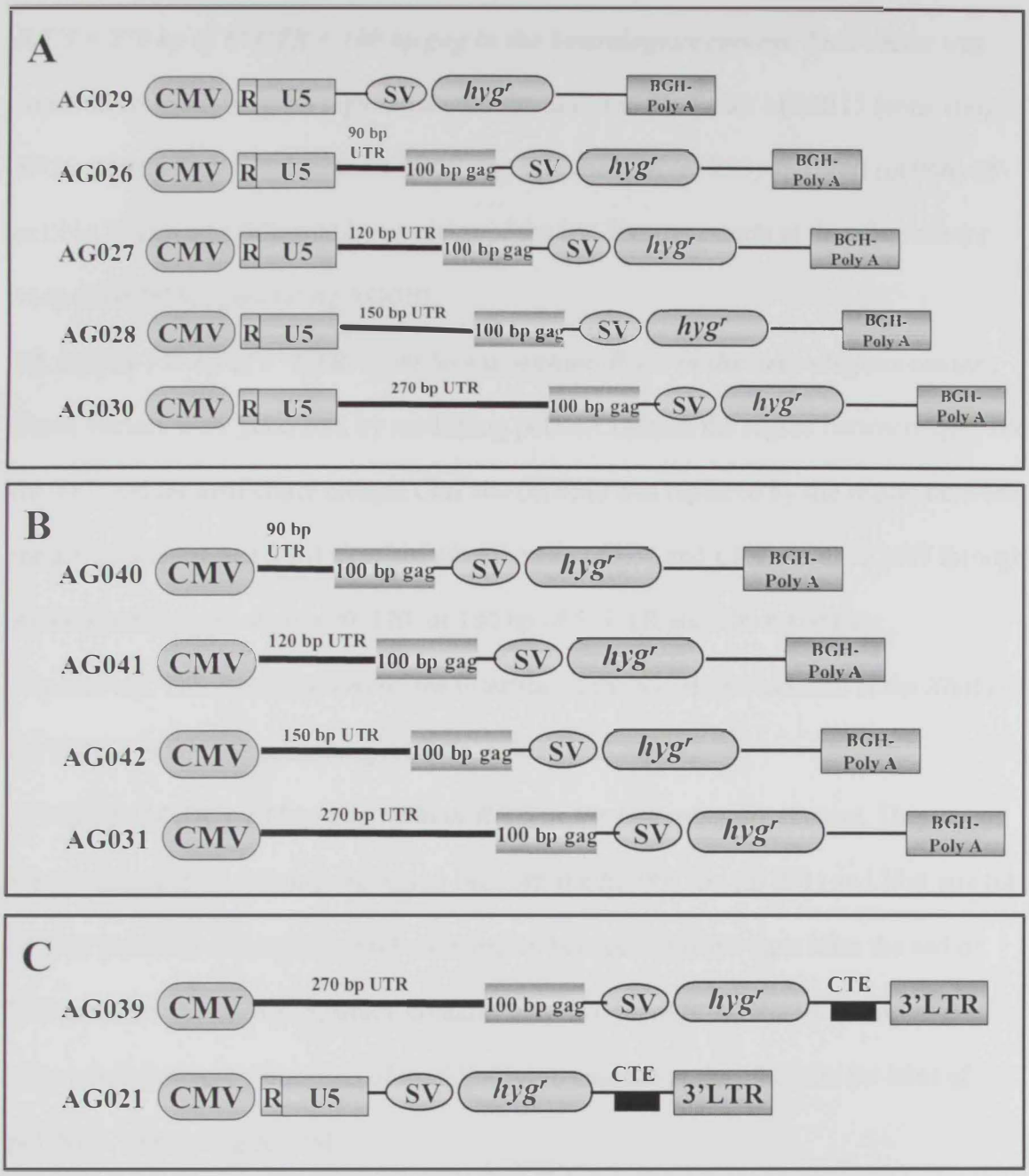

Figure II.4. Schematic representation of constructs testing the effect of R/U5 region on packaging. A) Transfer vectors with R/U5 region in heterologous context. B) Transfer vectors lacking the R/U5 region in the heterologous context. C) Transfer vectors with or without R/U5 region in the homologous context. CMV, human cytomegalovirus promoter-enhancer; SV, simian virus 40 early promoter; *hyg*^{r}, *hygromycin B phosphotransferase* gene; CTE, constitutive transport element of MPMV.

R/U5 + 270 bp of 5' UTR + 100 bp gag in the heterologous context. This vector was constructed by cloning the region between the *Spe*I and *Not*I sites of MB15 (consisting of R/U5, 270 bp of 5' UTR and 100 bp of *gag*) into the *Spe*I (nt 250) and *Not*I (nt 966) of pcDNA3. This was followed by insertion of the SV-Hygro cassette at the *Xba*I site (nt 984) of pcDNA3, generating AG030.

90, 120, or 150 bp of 5' UTR + 100 bp gag without R/U5 in the heterologous context. These vectors were generated by modifying pcDNA3 where the region between *Kpn*I site (nt 900) and the artificially created *Cla*I site (nt 951) was replaced by the region between the artificially created *Kpn*I site (just after the end of U5) and *Cla*I site of AG007 through AG009 containing only the 90, 120, or 150 bp of 5' UTR and 100 bp of *gag,* respectively. This was followed by the insertion of the SV-Hygro cassette at the *Xba*I site (nt 984) of pcDNA3, generating AG040, AG041 and AG042.

270 bp of 5' UTR + 100 bp gag without R/U5 in the heterologous context. This vector was constructed by deleting the region between the *EcoR*V site (nt 951) and *Not*I site (nt 966) of pcDNA3 and replacing it by the region between *Sfo*I site (just after the end of U5) and *Not*I site of MB15, which contains only 270 bp of 5' UTR and 100 bp of *gag*. This was followed by insertion of the SV- Hygro cassette at the *Xba*I site (nt 984) of pcDNA3, generating AG031.

R/U5 in the homologous context. This vector was constructed by deleting the region between the *Sfo*I site (nt 359 of FIV just after the end of U5) and *Not*I site of MTB, thus deleting the entire 5' UTR and religating the backbone on its own, generating AG021 which now contains only the R/U5 region in the presence of the SV-Hygro cassette and the 3' FIV LTR.

270 bp of 5' UTR + 100 bp gag without R/U5 in the homologous context. This vector was made through several steps of cloning in which the region between *Sfo*I (nt 359, just after the end of U5) and *EcoR*I of MB15 was subcloned into the *EcoR*V and *EcoR*I sites of pIC19H. The same region was next lifted by digesting the subclone with *Not*I and partial *Sac*I digest and ligated into the same sites of MB23, followed by insertion of the SV-Hygro cassette at the *Cla*I site, generating AG039.

Transfer vectors testing the 3' LTR. To study the effect of 3' LTR on packaging, different constructs were made to determine whether it has any contribution to the encapsidation of FIV RNA into the virus particles (Fig. II.5).

3' LTR in the homologous context. This vector was made by deleting the region between the *Spe*I and *Not*I sites of MTB and religating the backbone on its own, removing all the 5' *cis* acting sequences, generating AG001.

U3/R/U5 in the heterologous context. This vector was made by PCR amplifying the U3/R/U5 region using pCMV/RU5 as the template, OTR657 as the sense primer (S; 5' gcggatccTGGGATGAGTATTGGAACCCT 3'; nt 9120-9140 of FIV; FIV sequences are shown in upper case while lowercase denotes artificial sites created for cloning purposes) and OTR642 as the antisense primer (AS; 5' ccatcgatTGCGAAGTTCTCGGC CCGGATTC 3'; nt 9474- 9452 FIV). The resulting PCR product was digested with *BamH*I and *Cla*I and cloned into the same sites of pcDNA3, generating AG051.

U3/R in the heterologous context. This vector was made by PCR amplifying the U3/R region using pCMV/RU5 as the template, OTR657 as the sense primer and OTR658 as the antisense primer (AS; 5' ccatcgatGGTTCAATCTCA AATATTTATTG 3'; nt 9409-

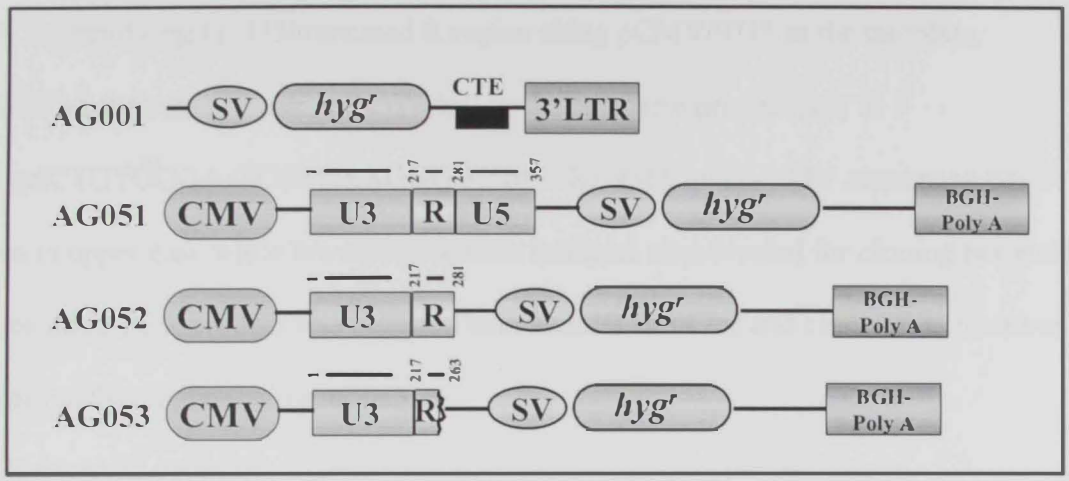

Figure II.5. Schematic representation of transfer vectors testing the effect of 3' LTR on packaging. The numbers represent the regions of FIV strain 34TF10 retained in the vectors. CMV, human cytomegalovirus promoter-enhancer; SV, simian virus 40 early promoter; *hygr*, *hygromycin B phosphotransferase* gene; CTE, constitutive transport element of MPMV.

9387 of FIV; FIV sequences are shown in upper case while lowercase denotes artificial sites created for cloning purpose). The resulting PCR product was digested with *BamH*I and *Cla*I and cloned into the same sites of pcDNA3, generating AG052.

U3/ truncated R (just before poly A) in the heterologous context. This vector was made by PCR amplifying the U3/truncated R region using pCMV/RU5 as the template, OTR657 as the sense primer and OTR659 as the antisense primer (AS; 5' ccatcgatCTGTGGGA GCCTCAAGGG 3'; nt 9383-9366 of FIV, FIV sequences are shown in upper case while lowercase denotes artificial sites created for cloning purpose). The resulting PCR product was digested with *BamH*I and *Cla*I and cloned into the same sites of pcDNA3, generating AG053.

Transfections and Infections of Cells

293T and Hela T4 cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 7% calf serum, respectively, from Hyclone (Logan, UT). 293T cells were transfected using the calcium phosphate method as described in Mustafa et al., 2005. Briefly, the cells were plated into 6-wells plates at a concentration of 4×10^5 cells per well a day before the transfection. The next day, media was changed 2 hours before the start of transfections. A DNA cocktail was prepared with 3 μg of each DNA in the case of transfections with three plasmids, and around 4.5 μg of each DNA in the case of two plasmids. As a control for transfection efficiency, the firefly luciferase expression vector, pGL3 control (Promega, Madison, WI), was used at a concentration of 250 ng/well. Plasmid DNA was diluted in a DNA cocktail buffer (250 mM $CaCl_2$, 150 mM NaCl, 10 mM Tris-Cl, pH 7.4, and 1 mM EDTA) and calcium

phosphate precipitate was formed using the 2X Transfection Buffer (50 mM HEPES, 180 mM NaCl, 4 mM sodium phosphate, pH 7). The precipitate was formed by adding the DNA cocktail to the 2X Transfection Buffer while bubbling, followed by vortexing and incubation at room temperature for 30-40 minutes. After the incubation, the precipitate was added to the 293T cells and incubated for 4 hours at 37°C. Transfections were stopped by washing the cells twice with PBS, followed by the addition of fresh media.

The media was changed the next day and lowered to 1.5 ml per well. In case of infections, virus released in the supernatants was harvested 72 hours post transfection and used to infect HeLa T4 cells using DEAE Dextran (10 mg/ml). Hygromycin resistant colonies were selected for 10-12 days, stained with crystal violet dye, and counted (Browning et al., 2001).

Luciferase Assays

To determine the transfection efficiency in the transfected cultures, a portion of the cells were used to detect the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) and Turner TD-20e luminometer (Turner Designs, Inc. Sunnyvale, CA). Briefly, transfected cells from a 6-well plate were trypsinized, washed once in cold PBS, and resuspended in 1 ml cold PBS. One tenth of these cells were used to prepare cell lysate using 100 µl of 1X Passive Lysis Buffer (Promega). Cell membranes were disrupted via three cycles of freeze/thaw (2 minutes in dry ice/2 minutes in 37°C water bath). Cellular debris was pelleted via microcentrifuging the lysates at 4°C for 5 minutes, followed by transfer of the clarified lysates to new eppendorf tubes. A portion of the lysate was used subsequently in the luciferase assay using 25 µl of the LARII reagent

provided in the kit. The luciferase readings were performed using a 5 seconds delay and 20 seconds integration time. Values obtained from the luciferase assay were normalized to the amount of protein used and these values were finally used to normalize the values obtained in the various assays including virus titration assay (colony forming units/ml) as well as packaging efficiencies (absorption units) determined from the Southern blots. The protein concentrations in the lysates were determined using the protein assay reagent (BioRad, Hercules, CA). Briefly, 2 μl of concentrated or 1:10 dilution of the cell lysate was tested in the protein assay against a standard curve prepared from BSA ranging from 0 μg to 16 μg protein.

Virus Isolation

The supernatants collected from the transfected cells were clarified away from the cellular debris by low-speed centrifugation at 4000 rpm for 30 minutes. This was followed by pelleting the viral particles by ultracentrifugation using SW28 rotor at 24,000 rpm for 2 hours at 4°C. Viral pellets were resuspended in 125 μl of TNE buffer (50 mM Tris-Cl, pH7.4, 100 mM NaCl, and 1 mM EDTA, pH 8.0). One third (~ 40 μl) was saved for analysis of virus particles by Western blots, while the remaining two-thirds (~ 80 μl) was used to isolate virion RNA using the Trizol LS reagent (Molecular Research Center, OH).

Nucleocytoplasmic Fraction of Transfected Cells

To isolate cytoplasmic RNA, 90% of the trypsinized transfected cells were resuspended in cold diethylpyrocarbonate (DEPC)- treated RLN buffer (50 mM Tris pH 8, 140 mM

NaCl, 1.5 mM $MgCl_2$) supplemented with 0.5 % NP40 and incubated on ice for 5 minutes to gently lyse the cells without disrupting the nuclei. The intact nuclei were gently spun down at 300X *g* for 2 minutes and 70% of the cytoplasmic fractions were carefully moved to fresh eppendorf tubes containing 1 ml Trizol reagent for subsequent RNA isolation.

RNA Isolation

Cytoplasmic and viral RNAs were isolated from the transfected cells 72 hours post transfection using the Trizol Reagent (Invitrogen Life Technologies). Virions in TNE buffer were lysed in 500 µl of Trizol LS reagent containing 8 µl of polyacryl (used as a carrier) prior to RNA isolation, while 90% of the transfected cells were lysed in 1 ml of Trizol. Briefly, cytoplasmic or viral lysates in Trizol were incubated at room temperature for 5 minutes followed by the addition of chloroform and vigorous shaking for 15 seconds and incubation at room temperature for 2-3 minutes. Phases were separated by centrifugation at 12000X *g* for 15 minutes, and 90% of the aqueous phase (clear in colour) was transferred to fresh eppendorf tubes containing 500 µl of isopropanol to precipitate RNA. The RNA was precipitated by mixing and incubating the tubes at room temperature for 10 minutes and centrifugation at 12000X g for 10 minutes to pellet down the RNA. The pellets were washed once with 70 % ethanol and centrifuged at 7500X *g* for 5 minutes. Pelleted RNA was resuspended in nuclease-free water, its concentration determined by spectrophotometry, and stored at –80 till further use.

RT-PCR

2.5 μg of cytoplasmic or one fifth of viral RNA was DNased-treated using 2-3 units of amplification grade DNase I (Invitrogen Life Technologies) at 37° C for 1 hour. DNase was heat inactivated at 65°C for 10 minutes after the addition of EDTA to 2.5 mM. One μl of the DNased RNAs were tested in PCRs using hygromycin-specific primers (Table II.1) to confirm the absence of DNA prior to cDNA synthesis. This was followed by cDNA synthesis by first denaturing the RNA in the presence of dNTPs and random hexamer primer for 5 minutes at 65°C, followed by quick cooling on ice for 5 minutes. After spinning the components to the bottom of the tube, cDNAs were made by using reverse transcription reactions set up with 100 units of Moloney Murine Leukemia virus reverse transcriptase (Invitrogen Life Technologies) in the presence of 40 units of RNasin RNase Inhibitor (Promega) for one hour at 42 °C. cDNAs were amplified using the SuperMix PCR cocktail (Invitrogen Life Technologies) supplemented with additional $MgCl_2$ or Taq polymerase (Applied Biosystems, California, USA). The PCRs for unspliced actin were performed as a multiplex in the presence of primer competimers for 18 S ribosomal RNA as a control for cDNA addition (18S Quantum competimer control, Ambion, TX).

PCR reactions were performed for 20-30 cycles at various annealing temperatures depending upon the melting temperature of the primer set used. The reactions were performed using the Perkin-Elmer 9700 thermocycler with MicroAmp tubes. Products of PCR were analyzed on 2-4% agarose gels in the presence of ethidium bromide. The gels were photographed and scanned using the Biometra gel documentation system (Whatman Biometra, Gottingen, Germany). Some of the gels were further processed for Southern

Table II.1: Primers used in RT-PCR and PCRs

Primer name	Sequences	Description
OTR366 (+)	5' CGGCACTTTGCATCGGCC 3'	nt 423-440, *hygromycin* gene
OTR367 (-)	5' CGGCGATCCTGCAAGCTC 3'	nt 1010-993 h*ygromycin* gene
OTR537 (+)	5' CATGTTTGTGATGGGTGTGAACCA 3'	nt 983-1005 of GAPDH
OTR538 (-)	5' GTTGCTGTAGCCGTATTCATTGTC 3'	nt 427-405 of GAPDH
OTR580 (+)	5' TGAGCTGCGTGTGGCTCC 3'	Spliced actin mRNA-specific primer (S) (Tan et al., 1995)
OTR581 (-)	5' GGCATGGGGGAGGGCATACC 3'	Spliced or unspliced actin mRNA-specific primer (A) (Tan et al., 1995)
OTR 582 (+)	5' CCAGTGGCTTCCCCAGTG 3'	Unspliced actin mRNA-specific primer (s-1) (Tan et al., 1995)
OTR600 (+)	5' GAACCCTGTCGAGTATC GTG 3'	nt 286 - 306 of FIV 5' LTR
OTR601 (-)	5' CTGGTTGCTGACTAATTGAG 3'	nt 1840-1821 of pCDNA3 or nt 244 -263 of TAR SV40
OTR660 (+)	5' GAGGACTTTTGAGTTCTCCCTTGAGGC 3'	nt 230-256 of FIV

OTR 459 (-)	5' aaaaaagcggccgcACTGCGAAGTTCTCGGCCCGG 3' *	FIV nt 357-337 (immediately before pbs).	
OTR 467 (+)	5' aaaaaagcggccgcGTTGGCGCCCGAACAGGGACT 3' *	nt 356-376 of FIV 34TF10	**90 bp Probe**
OTR662 (-)	5' AGCAGGAGTTCTGCTTAACAGCTTTC 3' *	nt 440-415 of FIV	
OTR627 (+)	5' TGGGATGAGTATTGGAACCC 3'	nt 1-20 of FIV Petaluma 5' LTR	**U3 Probe**
OTR517 (-)	5' gggcccaaattcgaaCTCGAAGTTTCACAAAGCACTGGT 3' *	nt 9335-9312 of FIV 34TF10 3' LTR	
OTR574 (+)	5' aaaaaagcggccgcATGGGGAATGGACAGGGGCGA 3' *	nt 628-648 of FIV *gag*	***gag* Probe**
OTR503 (-)	5' aaaaaagcggccgcTCCCTTCTCCAAATTTTTTACTCTTCC 3'*	nt 727-701of FIV gag	
OTR 516 (+)	5' ttcgaatttgggcccGAGTCTCTTTGTTGAGGACTTTTG 3' *	nt 9336-9359 of FIV 34TF10 3' LTR	**R/U5 Probe**
OTR460 (-)	5' aaaaaagcggccgcAAGTCCCTGTTCGGGCGCCAA 3' *	nt 377-357 of FIV 34TF10	

* FIV sequences are shown in uppercase while lowercase denotes sequences used for cloning purposes

blot analysis. The primers used in the PCR amplifications are listed in Table II.1.

Southern Blot Analysis

The gels designated for Southern blotting were prepared for capillary transfer overnight in 20X SSC (3 M NaCl/0.3 M sodium citrate) by denaturation (2X for 20 minutes at room temperature in 1.5 M NaCl/0.5 N NaOH) and neutralization (2X for 20 minutes at room temperature in 1.5 M NaCl/0.5 M Tris-Cl) using Hybond N+ membranes (Amersham Biosciences, UK). The next day, the membranes were cross-linked at 70,000 micro-joules (UV crosslinker BLX/254, Life Technologies) and hybridization performed by using the AlkPhos Direct Labeling Kit (Amersham, Arlington Heights, IL). Probes for hybridization were generated by PCR amplification and gel purification of the probe fragment (Qiagen gel extraction kit,) followed by their quantitation on agarose gels. The DNA fragments were labeled with alkaline phosphatase as specified in the kit. Briefly, 250-300 ng of purified DNA fragment was boiled for 5 minutes in a vigorously boiling water bath and snap frozen on ice for 5 minutes. Denatured DNA was spun down by brief (5 seconds) microcentrifugation and conjugated to alkaline phosphatase as per manufacturer's directions. The blots were prehybridized at 55°C in a pre-warmed hybridization buffer for 30 minutes to 1 hour followed by addition of extra hybridization buffer containing the probe. Blots were hybridized overnight at 55°C with gentle shaking, washed twice at 55°C with primary wash buffer (2M urea, 1% SDS, 50mM Na phosphate pH 7.0, 150 mM NaCL, 1 mM $MgCl_2$, 0.2 % blocking reagent), for 10 minutes and twice at room temperature with 1X secondary wash buffer (0.1 M Tris base, 0.2 M NaCl, pH 10.0) for 5 minutes and developed using the CDP-Star chemiluminescent detection

reagent. The blots were exposed to Fuji Medical X-ray film (HR-G30, Germany) for various lengths of times, digitized using the Biometra gel documentation system (Whatman Biometra, Gottingen, Germany), and analyzed for optical densities using the BioDoc Analyze software version 2.0.

Western Blot Analysis

Viral proteins were analyzed by Western blot analysis using a polyclonal antisera (QB2 fraction) from cats infected with the Petaluma strain of FIV (kindly provided by Dr. Ellen Collisson, Texas A&M University, College Station, TX). Briefly, virus particles in the TNE buffer were lysed in an equal volume of 2X SDS buffer along with betamercaptoethanol (β-me), boiled for 4 minutes, and loaded onto 8% SDS-polyacrylamide gels. The proteins were electrophoresed at 120 V for 1 hour and 45 minutes and transferred overnight using wet transfer buffer onto Hybond-P membranes (Amersham, Biosciences). The filters were blocked for one hour at room temperature in 5% dried milk (Regilait, 0% Fat, France) in 1X PBS-0.1% Tween buffer, followed by incubation in the polyclonal primary antibody QBT2 (1:2,500 dilution in PBS-Tween-1% milk) for 1 hour at room temperature. The blots were then washed three times in PBS-0.1% Tween for 5 minutes each, followed by incubation in the secondary antibody (goat anti-cat HRP) at 1:10,000 dilution for 1 hour at room temperature. The blots were washed three times using PBS-0.1% Tween buffer and developed using the Super Signal West Pico Chemiluminescent Substrate kit (PIERCE, Rockford, IL) and exposed to Fuji Medical X-ray film for various lengths of time.

Secondary RNA Structural Analysis

The folding potential of the various FIV sequences studied was tested by using the RNA structure sequence analysis software (version 3.6) (Mathews et al. 1999) that uses algorithms based on free energy minimization to predict the secondary RNA structure.

Chapter III

RESULTS & DISCUSSION I

Role of sequences in between the core packaging determinants in FIV RNA packaging

Our previous deletion analysis studies have revealed the presence of two core sequence elements within the 5' end of the FIV genome that contribute significantly to FIV RNA packaging (Fig. III.1). The first region consists of sequences from the start of the FIV transcript in the "R" region to within the first ~120 bp of 5' UTR and the second region consists of the first 100 bp of *gag* (Browning et al., 2003 a & b). However, it has not been clear what was the contribution of sequences in between these core elements towards packaging. Were these sequences completely dispensable or perhaps necessary to maintain any putative secondary RNA structure that the core region may fold into. Therefore, starting with our previously defined control vector, MB15 (Fig. III.2), that contains the R/U5, the entire 270 bp of the 5' UTR, and 100 bp of *gag*, we generated a new series of transfer vectors, AG002-AG004, that maintained the R/U5 and 100 bp of *gag*, but kept only the first 90, 120 or 150 bp of the 5' UTR, thus generating deletions of 180, 150, and 120 bp, respectively, between the core packaging determinants (Fig. III.2). In a second series of vectors, AG013-AG015, the deletions were replaced with heterologous sequences of same lengths from an expression vector, pcDNA3, to further determine whether the deleted/substituted region had a role at the structural level, perhaps as a spacer (Fig.III.2).

The two series of deletion and deletion/substitution vectors were tested for their ability to be packaged by the FIV proteins using our *in vivo* packaging assay (Fig. III.3) (Browning et al., 2001). Briefly, the assay consisted of MB22, a packaging construct that provides the FIV gag/pol structural proteins and MD.G, a VSV-G Env expression

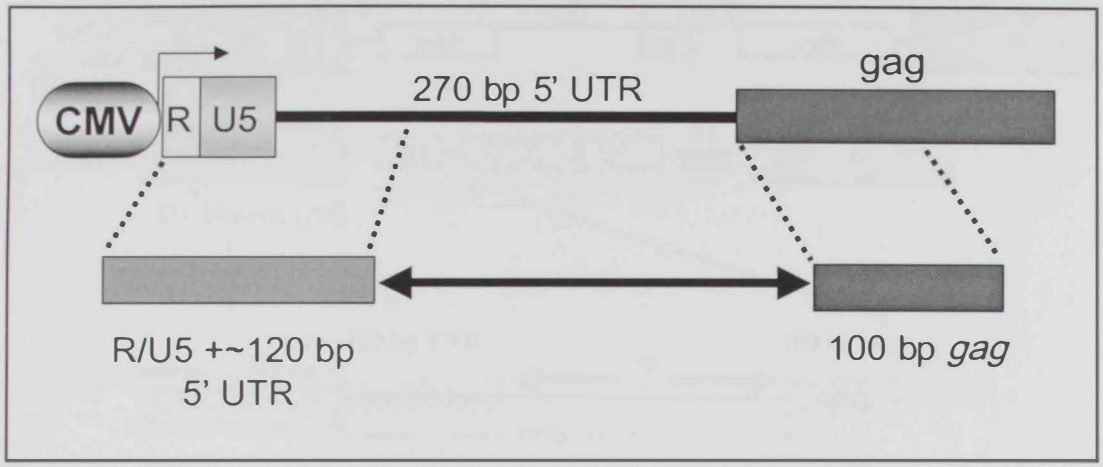

Figure III.1. Two discontinuous regions define the core packaging determinants of FIV. The first region consists of sequences from the start of the FIV transcript in the "R" region to within the first ~120 bp of 5' UTR and the second region consists of the first 100 bp of *gag* (Browning et al., 2003 a & b).

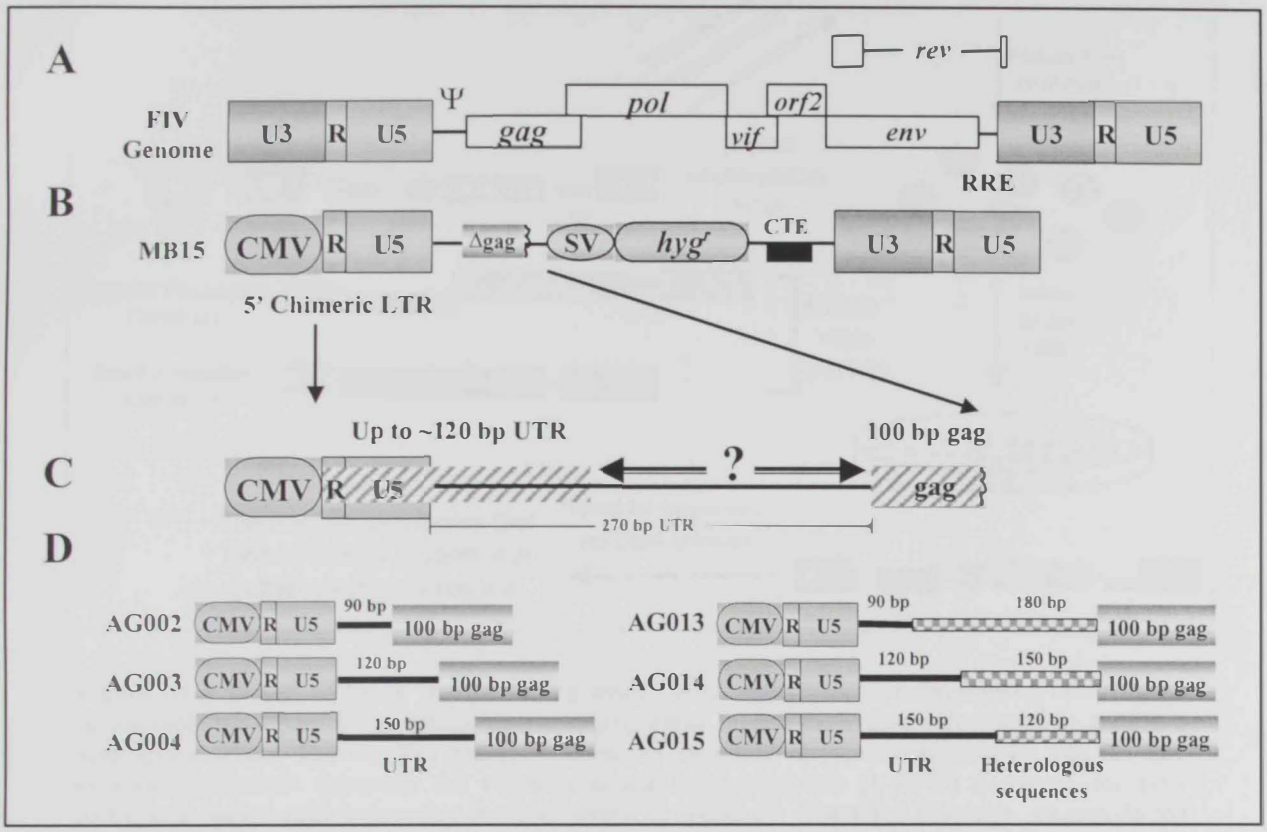

Figure III.2. Test of the region intervening the core packaging determinants for effects on packaging. Schematic representation of A) complete FIV genome, B) MB15, the control transfer vector, C) two core elements required for efficient RNA packaging. The arrow encompasses the sequences in between these two core element, and D) transfer vectors with deletions between the two core elements (AG002-AG004) and vectors with simultaneous deletions/insertions between the two core elements (AG013-AG015).

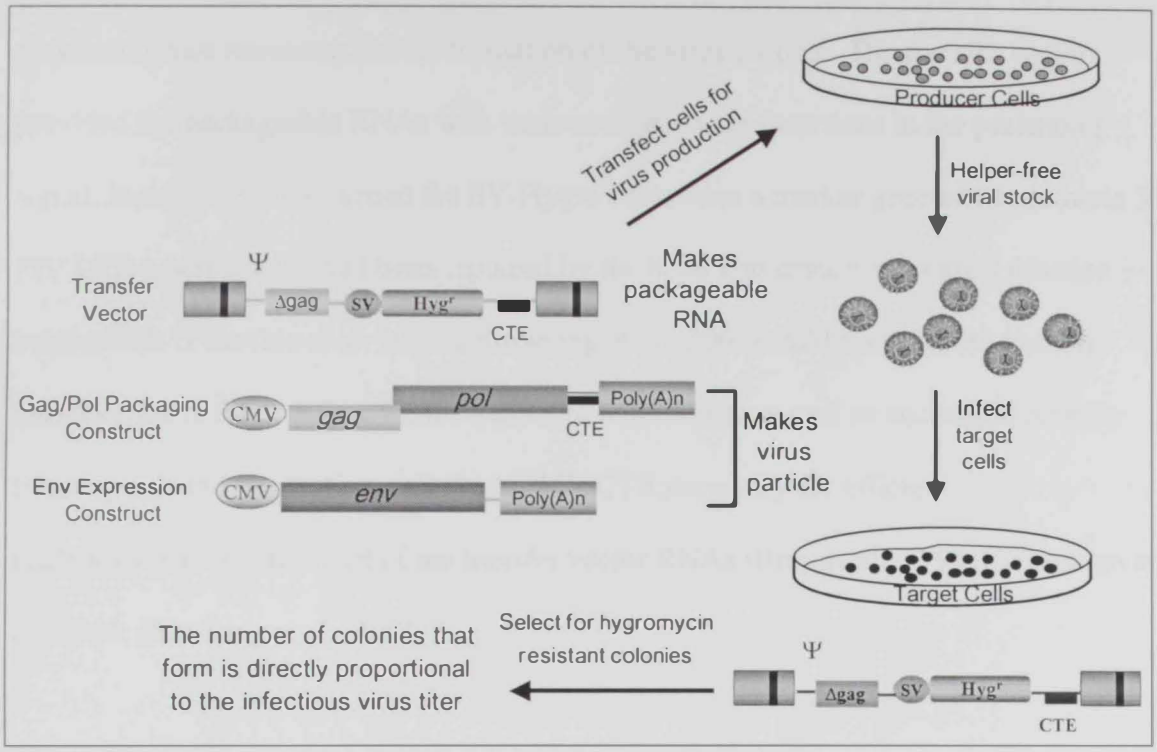

Figure III.3. Design of the *in vivo* packaging assay. RNA expressed from the transfer vector is the packageable RNA because it contains the packaging signal, ψ, and the hygromycin resistance gene as a selectable marker. The Gag/Pol packaging construct expresses the structural proteins, and the Env expression construct expresses the vesicular stomatitis glycoprotein (VSV-G) necessary for viral particles to infect cells expressing the corresponding receptor. The three plasmids are transfected together in the "producer" 293T cells that generate the "helper free" virus stocks containing the transfer vector RNA which is then used to infect target cells. Since these virions have only the transfer vector RNA as the genome, they are deficient for further rounds of replication, resulting only in a single round of replication. The successfully infected cells are selected by the use of hygromycin and the hygromycin-resistant colonies obtained are proportional to the virus titer .

plasmid that provides the *env* glycoproteins. Together, the two plasmids express the structural genes necessary for the formation of the virus particle. The transfer vectors provided the packageable RNAs with various deletions or insertions in the packaging signal. In addition, they carried the SV-Hygro cassette as a marker gene and a chimeric 5' FIV LTR where the U3 had been replaced by the hCMV to ensure successful function in human cells (Poeschla et al. 1998a; Browning et al., 2001). Additionally, the transfer vectors contain the 3' FIV LTR for transcript termination as well as successful reverse transcription and integration, and the MPMV CTE necessary for efficient nucleocytoplasmic transport of the transfer vector RNAs (Bray et al., 1994; Zolotukhin et al., 1994; Browning et al., 2001).

The region in between the two core packaging determinants is not necessary for packaging. The three-plasmid *trans* complementation assay was used to test the ability of the new series of transfer vectors to be packaged and propagated by FIV particles generated from MB22. Each transfer vector was co-transfected with MB22 and MD.G into 293T cells along with a luciferase expression vector to monitor transfection efficiencies in the transfected cells. Virus was harvested 72 hours post transfection and processed for viral RNA and proteins, as well as used to infect HelaT4 cells to determine the transduction efficiency of the packaged RNA. The infected cells were selected with media containing Hygromycin B to identify successfully transduced cells by the presence of hygromycin resistant colonies 10 days post infection. The transfected 293T cells were further fractionated into nuclear and cytoplasmic fractions for extraction of cytoplasmic RNA to determine successful transport of transfer vector RNAs, while whole cell protein

extracts were isolated for determination of transfection efficiencies.

First we analyzed the amount of transfer vector RNAs packaged into viral particles (Fig. III.4, panel A). Towards this end, RNA was extracted from two thirds of the pelleted virions, a portion of which was DNase-treated, reverse transcribed and used in PCR amplifications using primers across the R/U5 and SV40 promoter. Portions of the DNased RNA were tested by PCR to confirm the absence of plasmid DNA (Fig. III.4, panel A). To ensure that the amplifications were in the linear range of detection, PCRs were conducted for 20, 25, and 30 cycles, the resulting products were size fractionated on agarose gels, transferred onto nylon membranes, and hybridized with an R/U5 probe using a non-radioactive alkaline phosphatase labeling method (see Materials and Methods for details). The chemilluminescent signals emitted were captured on X-ray films for various lengths of time and digitized using the Biometra gel documentation system.

As observed in Fig.III.4, panel A, all six of the transfer vector RNAs with either deletions or deletion/substitution of the sequences in between the two core packaging determinants were packaged in the virus particles, however, with different efficiencies. This was despite the fact that the transfection efficiencies were within 2-fold of each other and all the cultures produced similar levels of viral particles (Fig. III.4, panels B & C). Since the PCR was conducted across the deleted or deleted/substituted region, the size of the PCR fragment varied with each construct, confirming that the correct packaging constructs were being expressed in each transfection.

To determine the packaging efficiencies of the various constructs accurately, the PCRs were repeated using primers within the R/U5 region that was common to all the

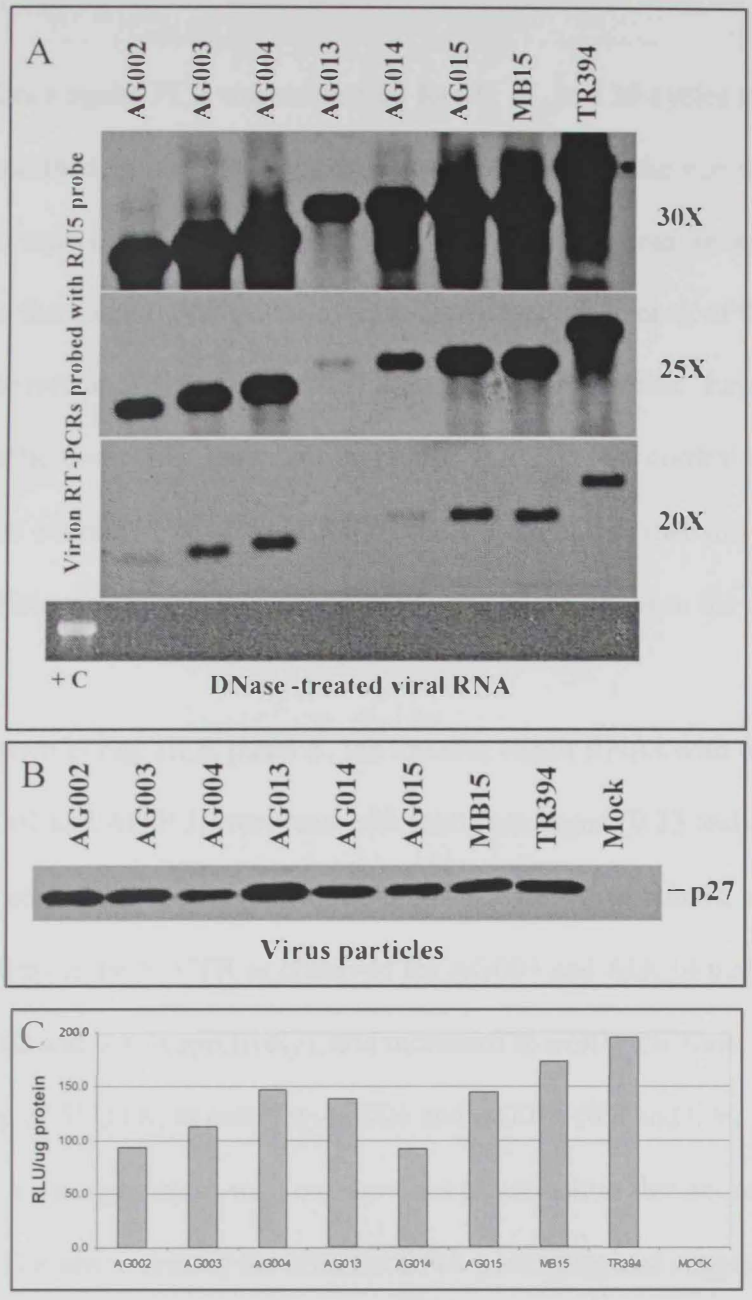

Figure III.4. Analysis of the relative packaging efficiencies of transfer vector RNAs with deletions and/or simultaneous insertions between the two core regions. A) Semi-quantitative RT-PCR of the viral cDNA amplified using OTR600 (R primer)/601 (SV40 primer) and probed with R/U5 probe. Bottom gel represents the DNase-treated viral RNA which was amplified using plasmid-specific primer OTR 366/367 (hygro primer), +C, plasmid as a positive control. B) Western blot analysis of equivalent amounts of the remaining one fourth portion of purified virus harvested from transfected cultures using the FIV antiserum from infected cats. C) Transfection efficiencies observed for different constructs assessed by the luciferase activity from the co-transfected pGL3 DNA using the Dual Luciferase Assay kit. RLU, relative light units.

constructs. Once again, PCR was conducted for 20, 25, and 30 cycles and Southern blotted, as described above. The optical densities observed in the various bands obtained on X-ray film were quantitated using the Biometra gel documentation system and normalized to the amount of luciferase expression observed in each of the transfected culture to take into account differences in transfection efficiencies. Finally, the transfection efficiencies obtained were compared to MB15, the control transfer vector with R/U5, the entire 270 bp of the 5' UTR, and 100 bp of *gag* that was assigned a packaging efficiency of 1 to allow for an easy comparison between the different constructs.

As shown in Fig. III.5, panel A, the transfer vector RNAs with only 90 bp of the 5' UTR, AG002 and AG013, were least efficiently packaged (0.23 and .06 respectively) when compared to MB15. The packaging efficiency increased substantially with the increase of 30 bp at the 5' UTR as observed for AG003 and AG014 that contain 120 bp of 5'UTR (0.62 and 0.4, respectively), and increased to nearly the same as MB15 by having 150 bp of 5' UTR, as seen for AG004 and AG015 (0.8 and 0.92 respectively). These results are in agreement with our previous observations that sequences around 120 bp of the 5' UTR are necessary for efficient RNA packaging and suggest that the deletions or deletions with simultaneous substitutions do not affect the packaging efficiency of FIV transfer vector RNAs substantially. Finally, the packaging efficiency observed for MB15 was comparable to that observed for TR394 (Fig. III.4), the wild type transfer vector that contains not only the entire 5' UTR, but also 333 bp of *gag* (Browning et al., 2001), confirming our earlier observations that along with part of the 5'

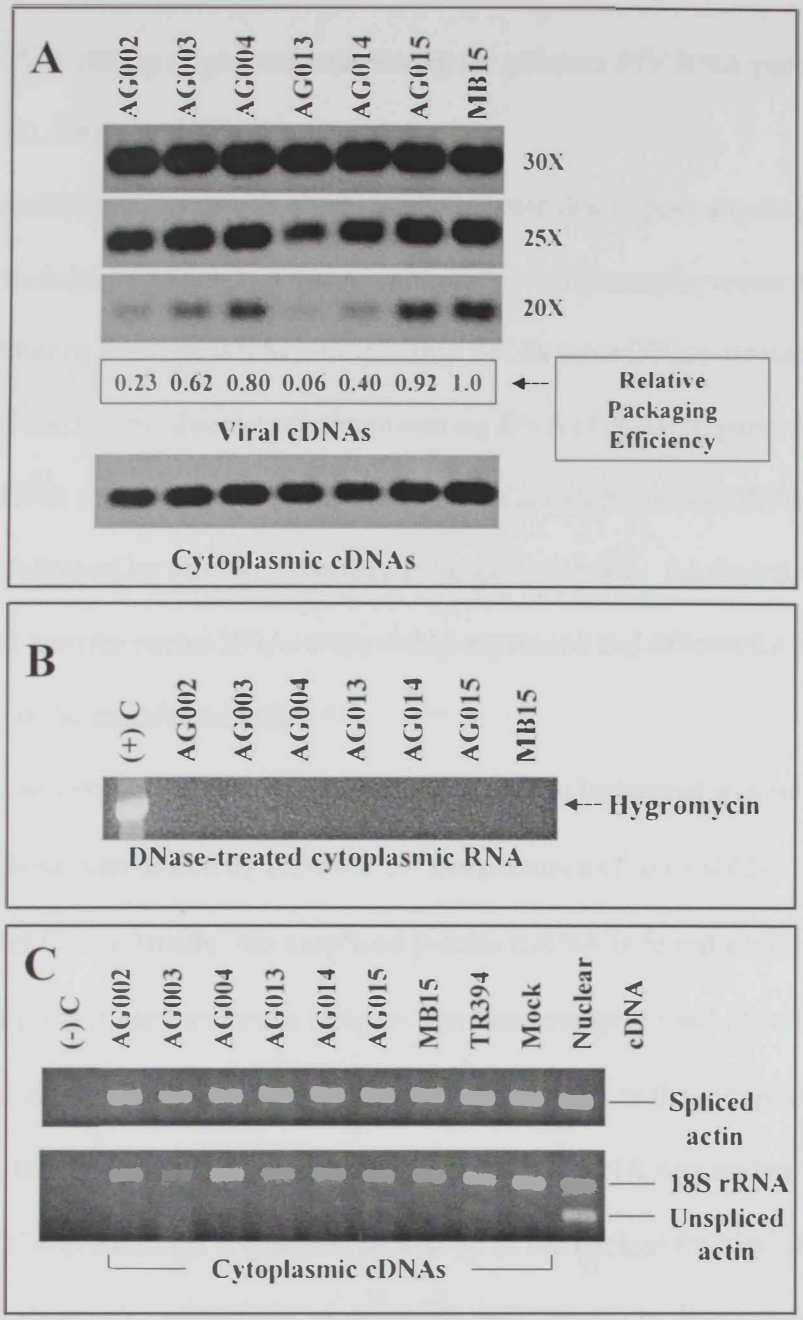

Figure III.5. The region in between the two core packaging determinants is not necessary for packaging. A) Semi-quantitative RT-PCR of the viral cDNA amplified using OTR 660/662 which gives the same size fragment and probed using the R/U5 probe. The relative packaging efficiency was calculated from average of ODs from 20 and 25 cycles. B) DNase-treated cytoplasmic RNA amplified using plasmid-specific primer OTR 366/367 (hygro primer) +C, plasmid as a positive control. C) Control for nucleocytoplasmic fractionation technique. The upper panel is the RT-PCR on cytoplasmic RNA for spliced actin which should be present in both the nuclear and cytoplasmic fractions, while the bottom panel is a multiplex RT-PCR for unspliced β-actin mRNA that should be exclusively nuclear and 18S ribosomal RNA as a control for the presence of cDNA.

UTR only the first 100 bp of *gag* were necessary for efficient FIV RNA packaging (Browning et al., 2003a and b).

It is possible that the results obtained above were due to poor expression, instability, or deficient nucleocytoplasmic transport of some transfer vector RNAs. Therefore, similar to the viral RNAs, cytoplasmic RNAs were DNase-treated and confirmed by PCR for the absence of contaminating DNA (Fig. III.5, panel B). This was followed by cDNA preparation and PCR analysis of the cytoplasmic cDNAs for 20, 25, and 30 cycles followed by Southern blotting as described above. As shown in Fig. III.5, panel A, all the transfer vector RNAs were stably expressed and efficiently transported to the cytoplasm of the transfected cells.

To ensure that our nucleocytoplasmic fractionation technique was reliable, the cytoplasmic RNAs were tested by RT-PCR for the presence of unspliced β-actin mRNA (Fig. III.5, panel C). Normally, the unspliced β-actin mRNA is found exclusively in the nucleus unless the nuclear membrane integrity has been compromised (Tan et al., 1995). As can be seen, only the spliced β-actin mRNA was observed in the cytoplasmic fractions (Fig. III.5, panel C, top half), while the unspliced RNA was undetectable with 30 rounds of PCR eventhough it could be picked up in the nuclear fraction (Fig. III.5, panel C, bottom half). To ensure that each cytoplasmic sample in the unspliced β-actin PCRs contained amplifiable cDNAs, the unspliced β-actin PCR was conducted in the presence of primers for 18S ribosomal RNAs as an internal control (Fig. III.5, panel C, bottom half).

The drastic effect on packaging observed for AG013 over and above the lack of 5' UTR sequences is probably due to the 180 bp heterologous sequences that were inserted

to maintain the spacing that any potential structural elements within the two core regions may assume. This 180 bp insertion, and in particular, the last 30 bp of it, seems to have destabilized the entire region since their removal (as in AG014 that contains only the first 150 bp of the 180 bp insertion) re-stabilized the region for packaging to nearly the same extent as AG003 and AG014 (vectors with 120 bp UTR with or without 150 bp insertions, respectively). Other than AG013, the packaging efficiencies between the vectors with the internal deletions were similar to the vectors where the deletions had been replaced by the heterologous sequences, suggesting that the sequences in between the core packaging determinants were not needed as spacers either and the core elements could fold into functional packaging determinants independently of the spacer region.

Finally, the ability of the transfer vector RNAs to transduce the hygromycin marker gene to target cells was determined by infecting HeLa cells with virions produced by the 293T cells. Colony numbers observed upon hygromycin selection were adjusted to the amount of firefly luciferase expression observed per microgram protein to normalize for transfection efficiencies. Overall, the transfer vector RNAs were transduced to the target cells with about 1- to 4-folds reduced efficiency than the control vector, MB15 (Table III.1). Transfer vector RNAs with the 5' UTR deletions, AG002-AG004 were propagated with ~ 2-3-fold reduced efficiency, while those with the deletion/ insertions, AG013-AG015 were propagated with a 1-4-fold reduction when compared to MB15 (Table III.1). The vectors with only 90 bp of the 5' UTR (AG002 and AG013) exhibited the greatest reduction in titers, probably reflecting the poor packaging efficiency observed with these vectors (see Figs. III.4 & III.5), while vectors with 120 (AG003 and AG014) and 150 bp UTR (AG004 and AG015), resulted in near wild type

Table III.1. The intervening region between the core packaging determinants does not affect transfer vector RNA propagation

Vector name	5' UTR / *gag* maintained	Heterologous sequences added	LUC Normalized Titer (CFU/ml)	Fold Reduction	P value (< 0.01)
AG002	90/100	-	713±68	3.1	0.0001
AG003	120/100	-	966±123	2.3	0.0056
AG004	150/100	-	910±76	2.4	0.0004
AG013	90/100	180	588±101	3.8	0.001
AG014	120/100	150	1,208±91	1.8	0.0001
AG015	150/100	120	1,646±66	1.3	0.002
MB15	270/100	-	2,229±18	1.0	-
TR394	270/333	-	2,417±238	0.9	0.064
Mock	No DNA	-	< 1	-	-

titers (within 1-2-folds) either with or without the insertions, reflecting the improved packaging efficiencies observed with these vectors.

Structural analysis of the folding potential of the 5' end of the FIV genome and its correlation with the packaging efficiency data

To determine how the folding of the spacer region in between the two core determinants was affected by the deletions or deletion/insertions, the regions between R and end of 100 bp *gag* of our various mutant vectors was folded using the RNAstructure (version 3.6) of Zuker and colleagues that uses energy minimization algorithms to predict foldings of RNA molecules (Mathews et al., 1999). Folding of this region in TR394 and MB15, the control vectors with the entire 270 bp UTR and either 333 or 100 bp of *gag*, revealed the presence of four stem loops (SL1-4) in U5/5' UTR region starting from about 20 bp into the U5 to about 6 bp downstream of the major splice donor, mSD (Fig. III.6, panels A & B). These stem loops seemed very stable since they were perfectly conserved in the four predicted structures in TR394 and the two predicted structures in MB15. Furthermore, the major splice donor in each case was downstream of the four stable stem loops and formed part of the stem that completed the folding of the stably folded region confirming the prior functional analysis that revealed that sequences downstream of mSD and upstream of *gag* did not contribute to packaging (see Fig. III.6) (Browning et al., 2003a).

SL2 is potentially the key packaging determinant of FIV. Folding of the vectors RNAs containing the incremental deletions or deletions with insertions revealed that most of the folding of the wild type UTR was destroyed in AG002/AG013 and AG003/AG014,

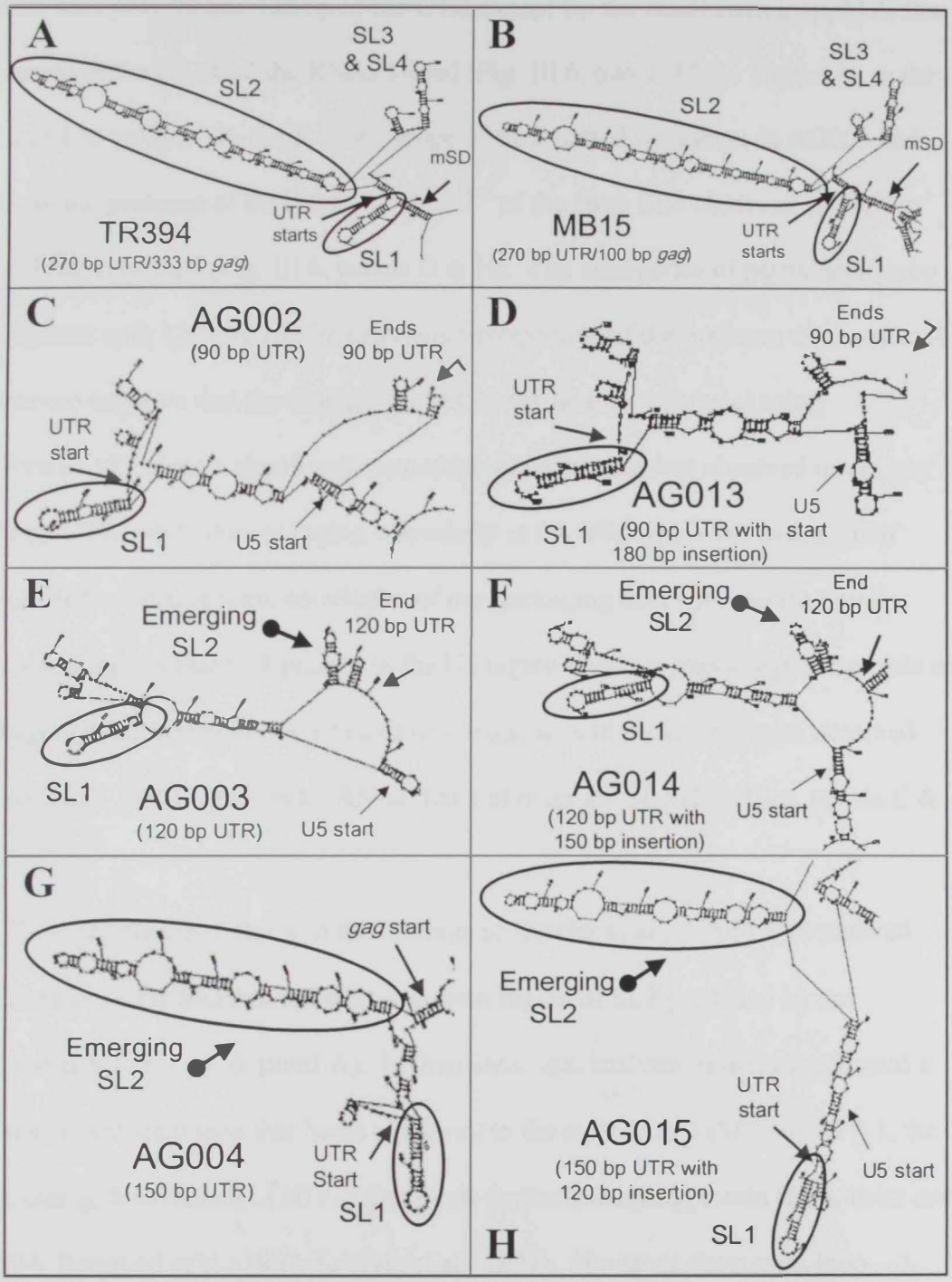

Figure III.6. The folding potential of the mutant transfer vectors either with deletions or deletions/insertions in between the two core elements. The ovals represent the conserved stem loop structures, SL1-4. The emerging SL2 loop observed in AG003/AG004 and AG014/AG015 is highlighted by the arrows with the knobs at the end.

the vectors with only 90 and 120 bp of the UTR except for the small stem loop, SL1, that was observed in the U5 of all the RNAs folded (Fig. III.6, panels C-F). However, in the presence of 120 bp of UTR, the first few loops of SL2 started to emerge in AG003 and 14, while in the presence of the150 bp UTR, 2/3rd of the large SL2 observed in TR394 and MB15 had reformed (Fig. III.6, panels G & H). The emergence of partial stem loop 2 in the vectors with 120 and 150 bp deletions (irrespective of the presence or absence of the insertions) suggests that the first 2/3^{rds} of SL2 may be the major packaging determinant of FIV since a significant restoration of packaging was observed in vectors with 120 bp UTR, while the packaging was nearly at the wild type level with 150 bp UTR (Fig. III.5). Furthermore, correlation of our packaging data with the structural analysis also suggests that SL1 present in the U5 region does not play a significant role as the packaging determinant in FIV since drastic reductions in packaging were observed with AG002 and AG013, the vector RNAs that still maintain SL1 (Fig. III.6, panels C & D).

These results are contrary to the findings of Kemler et al., (2002) who mutated what encompasses the fourth loop/bulge and stem region of SL2 predicted by our computer analysis (Fig. III.6, panel A). In their structural analysis, this region formed a small independent stem loop that had a similarity to the stem loop 3 (SL3) of HIV-1, the main packaging determinant of HIV-1 that binds the nucleocapsid protein (Berkowitz and Goff, 1994; Berglund et al., 1997; Lochrie et al., 1997). However, their stem loop abrogating mutations did not affect FIV RNA packaging. One reason for the contrary findings could be that the first three bulges/stems of SL2 may be the main determinants of FIV RNA packaging, which should not have been affected by their mutations (Fig.

III.6, panel A). This speculation is supported by the observation that maintenance of 120 bp UTR in AG003/AG014 resulted in the appearance of the first two of the bulges/stems of SL2 that restored ≥ 50 % of the packaging potential of transfer vector RNAs (Figs. III. 5 and III. 6, panels E & F). Thus, FIV may not need the entire SL2 for packaging, but only the first part of this structure that is probably sufficiently formed by the presence >120 bp in the UTR, but <150 bp.

Interestingly, the *gag* region (100 or 333 bp) in the control vectors did not fold into any particularly consistent stem loop structure. Either our analysis was not stringent enough to pick up any signature folding patterns, or *gag* may not necessarily be involved at the structural level. Since the functional data demonstrates that *gag* sequences are critical for packaging, the RNA folding data suggests that sequences in *gag* may help FIV differentiate between genomic and subgenomic mRNAs prior to packaging by providing intronic sequences to the packaging signal. Or, it is possible that sequences in *gag* may provide a more indirect role similar to that observed in HIV-2 related to a co-translational mechanism for identifying genomic mRNAs for encapsidation.

In short, using a combination of mutational analyses and computer predictions, studies presented in this part of the thesis clearly demonstrate that the FIV packaging determinants are found as two core discontinuous regions at the 5' end of the viral genome. The first of these regions (R/U5 + 150 bp UTR) is present upstream of the major splice donor and folds into a complex stem loop structure, while sequences in the first 100 bp of *gag* also contribute critically to the ability of the virus to recognize the genomic message from the plethora of cellular and spliced mRNAs.

These results are in partial agreement with those of Kemler et al., 2002 and 2004

who found that the R/U5 region (in particular), but also the 154 bp of the UTR harbored the main packaging determinants of FIV, and simultaneously required help from the first ~ 90 bp of *gag*. Since all our vectors contained R/U5, we could not differentiate between the relative contributions of vectors with and without R/U5 to packaging. However, based on the analysis of the structural data, our studies suggest that probably the main packaging determinant will lie more within the first 150 bp of the UTR than the R/U5 sequences.

Chapter IV

RESULTS & DISCUSSION II

Role of 5' and 3' LTRs on the packaging efficiency of FIV RNA.

Results presented in Chapter III clearly show the discontinuous and multipartite nature of the FIV packaging determinants, revealing at least two regions of the FIV 5' end that significantly affect FIV RNA in packaging. However, our earlier mapping studies had implicated other regions of the FIV genome towards packaging, including the 3' LTR (Browning et al., 2003 a &b). In addition, studies published around the same time also pointed to the role of 5' R/U5 sequences as being actually the primary packaging determinant (Kemler et al., 2002 & 2004). Since all our previous studies did not differentiate between the 5'UTR and the R/U5 region and were done in the presence of the 3' LTR, we wanted to determine the relative contribution of the FIV 5' and 3' LTRs to FIV RNA packaging.

Towards this end, we utilized a two-plasmid *trans* complementation assay that consisted of a packaging construct and a series of transfer vectors. Unlike the 3-plasmid system, this assay lacked the vector that would provide the envelope proteins since there was no need for the infection of target cells as most of the transfer vectors lacked the sequences necessary for reverse transcription, integration and propagation into the target cells. The transfer vectors contained various portions of the putative FIV packaging signal expressed from the hCMV promoter for transcript initiation and the BGH poly A signal for transcript termination.

R/U5 region increases the packaging efficiency of heterologous FIV transfer vector RNAs. To determine the role and contribution of the 5' R/U5 region to packaging, the expression vector pcDNA3 was modified to generate a series of transfer vectors that

contained the two core packaging determinants of FIV either in the presence or absence of R/U5 in the non-viral heterologous context (Fig. IV.1, panel A). To ensure that we had the entire core region, we maintained either 90, 120, or 150 bp of the 5' UTR and the first 100 bp of *gag*. AG026, AG027, and AG028 are transfer vectors that contain the core packaging determinants in the presence of R/U5, while AG040, AG041, and AG042 contain only the core packaging determinants (Fig. IV.1, panel A). Since we were testing such small parts of the FIV genome, the ~ 2 kb SV-Hygro cassette was inserted in the transfer vectors to increase the size of the packageable RNAs and make them relatively the same size for easier relative comparison.

As stated earlier, each transfer vector was co-transfected with MB22 into 293T cells, virus was harvested 72 hours post transfection and processed for RNA and proteins, while a portion of the transfected cultures were fractionated into nuclear and cytoplasmic fractions for isolation of cytoplasmic RNA. To monitor for variations in transfection efficiencies, the luciferase expression vector, pGL3 Control (Promega), was also included in the DNA cocktail and a portion of the transfected cultures was used to determine luciferase expression.

First, the expression of the transfer vector RNAs in the transfected cultures was analyzed to determine if the vector RNAs were expressed and efficiently transported to the cytoplasm. Towards this end, equal amounts of the cytoplasmic RNA from each transfected culture was DNase-treated, reverse transcribed, and PCR amplified using OTR 467/OTR503, primers within the PBS and the *gag* region (Fig. IV.1, panel B). As shown, all transfer vector RNAs were expressed efficiently in the cytoplasm of the transfected cells, demonstrating that the transfer vector RNAs were stable and

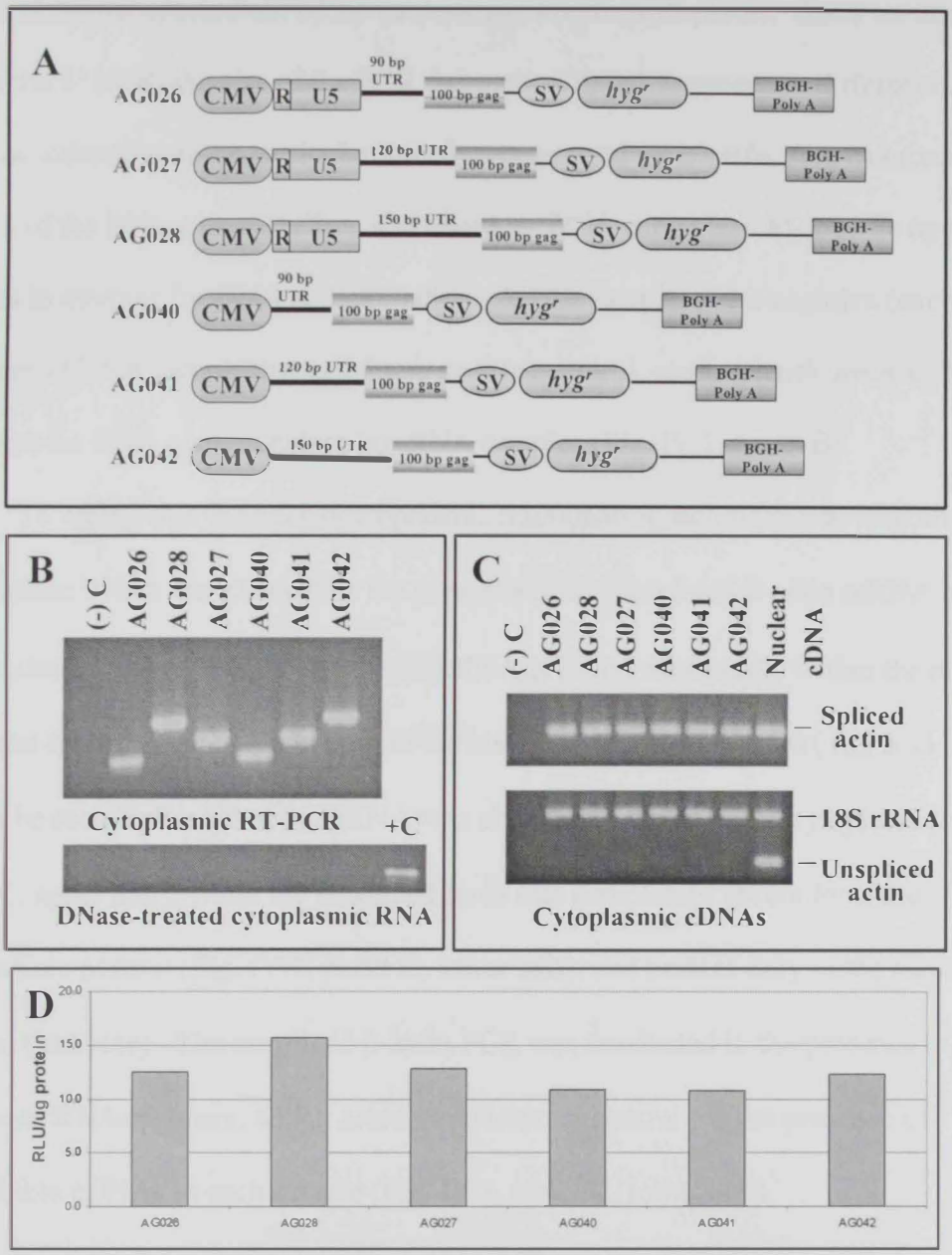

Figure IV.1. Test of the FIV R/U5 region towards contribution to FIV RNA packaging. A) Schematic representation of the transfer vectors with/without the R/U5 region in addition to the two core regions in heterologous context. B) RT-PCR of cytoplasmic cDNA amplified using OTR467 (pbs) and OTR503 (*gag*). The bottom gel shows the DNase-treated cytoplasmic RNA which was amplified using plasmid specific primer OTR366/367 (hygro primer), +C, plasmid as a positive control. C) Control for nucleocytoplasmic fractionation technique. The upper panel is the RT-PCR on cytoplasmic RNA for spliced actin which should be present in both the nuclear and cytoplasmic fractions, while the bottom panel is a multiplex RT-PCR for unspliced β-actin mRNA that should be exclusively nuclear and 18S ribosomal RNA as a control for the presence of cDNA. D) Transfection efficiency observed for different constructs assessed by the luciferase activity from the co-transfected pGL3 DNA using the Dual Luciferase Assay kit. RLU, relative light units.

successfully transported out of the nucleus and into the cytoplasm. Since we amplified across the 5' UTR, the size of the PCR fragment differed between the different constructs and was indicative of the particular transfer vector used for transfection. As a control, a portion of the DNase treated RNA was tested by PCR using plasmid-specific hygromycin primers to monitor for DNA contamination. All the samples were negative (except for the plasmid DNA sample that acted as a positive control, see last lane), assuring that there was no DNA contaminating our RNA samples (Fig. IV.1, panel B).

To ensure that the nucleocytoplasmic fractionation technique was effective, the cytoplasmic RNAs were tested for the presence of the unspliced β-actin mRNA by PCR. As mentioned earlier, unspliced β-actin mRNA is found exclusively within the nucleus, while the spliced form is found both in the nucleus and the cytoplasm (Tan et al., 1995). As can be seen, spliced β-actin mRNA was abundantly found in the cytoplasm (Fig. IV.1, panel C, upper half), while the unspliced form was completely absent form the cytoplasmic portion (Fig. IV.1, panel C, lower half), and present only in the nuclear fraction (last lane). The unspliced β-actin PCR was conducted in the presence of 18S ribosomal RNAs primers, which acted as an internal control for the presence of amplifiable cDNAs in each sample (Fig. IV.1, panel C, lower half).

Once confirmed that all transfer vector RNAs were efficiently expressed and transported to the cytoplasm, we went on to analyze the amount of RNA packaged into the actual virus particles. Similar to cytoplasmic RNAs, the viral RNAs were DNase treated, reverse transcribed and used in PCR amplifications. To make sure that the amplifications were in the linear range of detection, PCRs were conducted for 20, 25, and 30 cycles, the resulting products were run on agarose gels, transferred to nylon

membranes, and hybridized with an FIV *gag* probe using a non-radioactive alkaline phosphatase labeling method (see Materials and Methods for details). The chemilluminescent signals emitted were captured on X-ray films that were digitized and the optical density in the various bands quantitated using the Biometra gel documentation system. Finally, the optical densities obtained were normalized to the amount of luciferase expression observed in each of the transfected culture to take into account differences in transfection efficiencies.

As observed in Fig. IV.2, all transfer vector RNAs were packaged, but to various extent. The constructs with the R/U5 region (AG026-028) were packaged more efficiently showing relative packaging efficiencies (RPE) of 3.2, 6 and 10 than those lacking the R/U5 region (AG040-042) displaying relative packaging efficiencies of 1, 3.5 and 2.7. This is despite similar transfection efficiencies (Fig. IV.1, panel D) and the production of similar levels of viral particles in the transfected cultures (Fig. IV.2). In agreement with our previous results, the deletion construct with only 90 bp of UTR was packaged least efficiently (RPE of 3.2), followed by the construct with 120 bp UTR (RPE of 6), while 150 bp UTR enhanced packaging the most (RPE of 10). Together, these results reveal that efficient packaging by FIV proteins requires at most 150 bp of the 5' UTR in the presence of 100 bp of *gag*, and this level of packaging can be nearly doubled or more by the presence of R/U5 sequences at the 5' end.

Role of the 3' LTR on the packaging efficiency of FIV RNA. To study the role of the 3' LTR, three new transfer vectors were constructed. AG030 and AG031 represent transfer vectors that express the entire 270 bp UTR either in the presence or absence

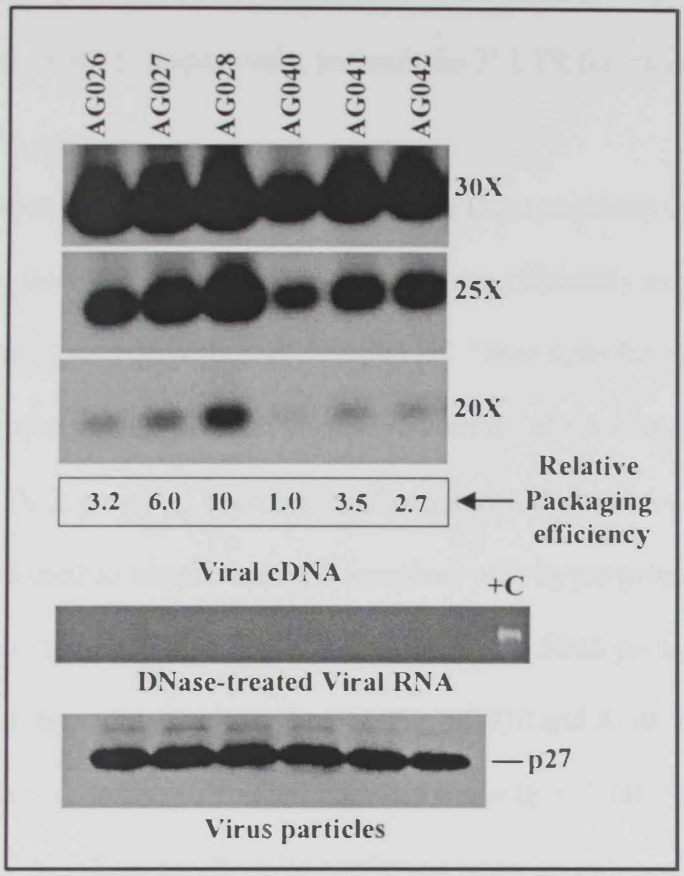

Figure IV.2. The R/U5 region increases the packaging efficiency of heterologous FIV transfer vector RNAs. Semi-quantitative RT-PCR of viral cDNA amplified using OTR467 (pbs) and OTR662 (90 bp of 5' UTR) for 20, 25 and 30 rounds of PCR and probed using the 90 bp probe. The panel below shows the DNase-treated viral RNA which were amplified using plasmid specific primer OTR366/367 (hygro primer). The bottom panel shows the Western blot analysis of equivalent amounts of the remaining one fourth purified virus harvested from transfected cultures using the FIV antiserum from infected cats. The relative packaging efficiency was calculated from average of ODs from 20 and 25 cycles.

of R/U5 sequences, while MB15 and AG039 contain the entire 270 bp UTR either in the presence or absence of R/U5, respectively, but with the 3' LTR for transcript termination (Fig. IV.3, panel A).

The 3' LTR contributes to FIV RNA packaging. The four constructs were tested in our packaging assay as described before. All constructs were efficiently expressed in the cytoplasm of the transfected cells (Fig. IV.3, panel B). These cytoplasmic RNA extracts were fractionated appropriately since no unspliced β-actin mRNA could be observed in the cytoplasm(Fig. IV.3, panel C), and no amplification could be observed when the DNased RNAs were used as template in PCR reactions with hygro primers (Fig. IV.3, panel B). Finally, semi-quantitative RT-PCR analysis of the RNA packaged in the virions revealed that the vectors without the 3' LTR, AG030 and AG031 were packaged with 5- and 3-fold less efficiency than their counterparts with 3' LTR, MB15 and AG039, respectively (Fig. IV.4). These results were confirmed using an independent primer pair (data not shown). These constructs also confirmed our earlier results that R/U5 increased the packaging efficiency of vector RNAs by ~ 2-3-folds despite the presence of the entire 5' UTR (compare AG030 with AG031 and MB15 with AG039) (Fig. IV.4). This was despite the fact that all vectors were transfected with similar efficiencies (Fig. IV.3, panel D) and similar amounts of virions were produced in each culture (Fig. IV.4).

Taking this analysis further, two more transfer vectors were constructed to determine whether 3' LTR could enhance the packaging efficiency of transfer vector RNAs in the absence of the 5' UTR. AG021 and AG029 contain the FIV R/U5 region at the 5' end and either the BGH poly A signal or the 3' FIV LTR downstream of the SV-

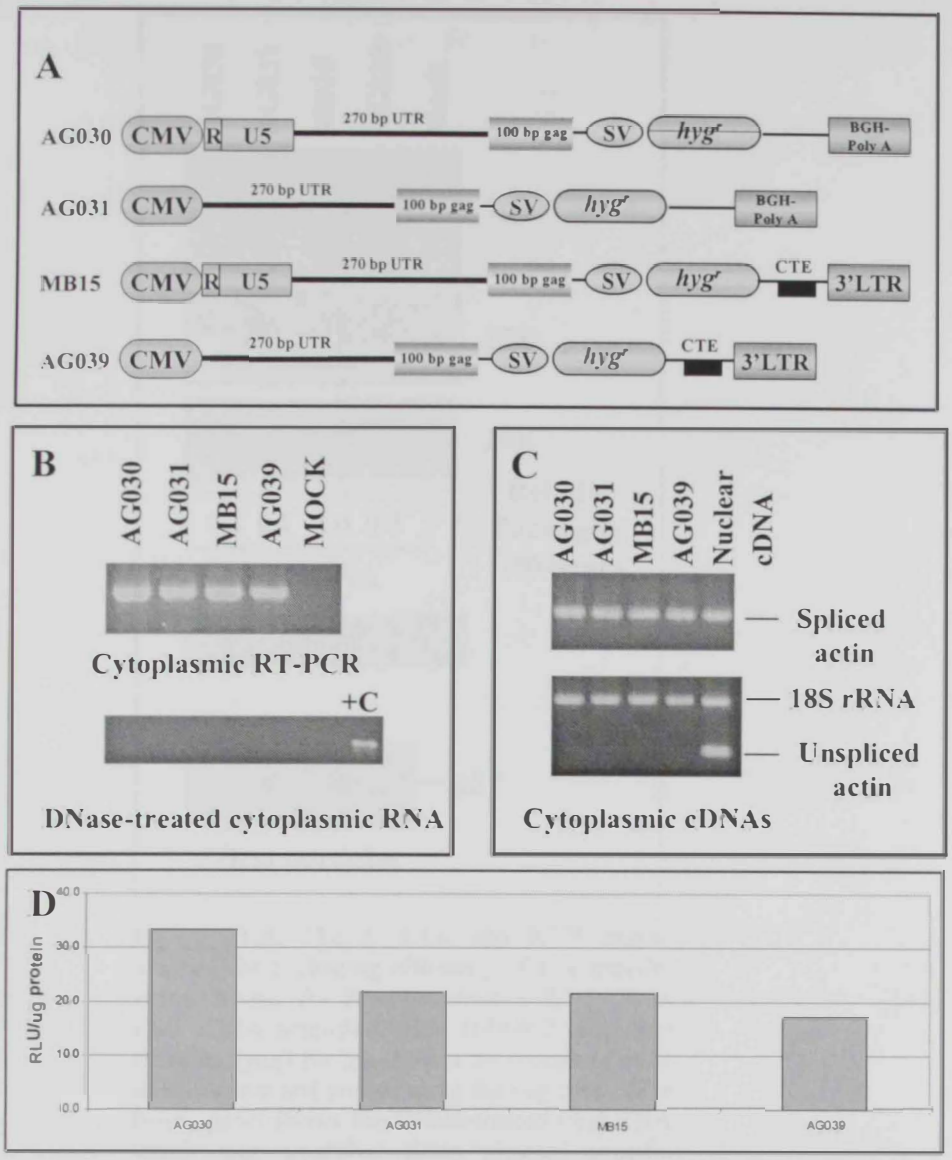

Figure IV.3. Test of the 3' LTR and R/U5 regions towards contribution to FIV RNA packaging. A) Schematic representation of the transfer vectors with/without the 3' LTR and R/U5 region in the presence of the entire 270 bp of 5' UTR and 100 bp of *gag*. B) RT-PCR of cytoplasmic cDNA amplified using OTR467 (pbs) and OTR503 (*gag*). The bottom panel shows the DNase-treated cytoplasmic RNA which was amplified using plasmid specific primer OTR366/367 (hygro primer). +C, plasmid as a positive control. C) Control for nucleocytoplasmic fractionation technique. The upper panel is the RT-PCR on cytoplasmic RNA for spliced actin which should be present in both the nuclear and cytoplasmic fractions, while the bottom panel is a multiplex RT-PCR for unspliced β-actin mRNA that should be exclusively nuclear and 18S ribosomal RNA as a control for the presence of cDNA. D) Transfection efficiency observed for different constructs assessed by the luciferase activity from the co-transfected pGL3 DNA using the Dual Luciferase Assay kit. RLU, relative light units

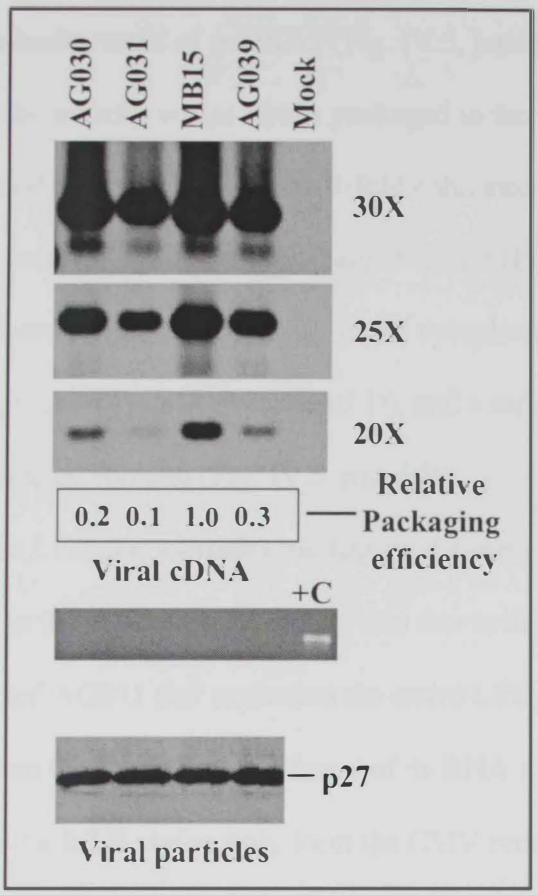

Figure IV.4. The 3' LTR and R/U5 region increase the packaging efficiency of FIV transfer vector RNAs. A) Semi-quantitative RT-PCR of viral cDNA amplified using OTR467 (pbs) and OTR503 (*gag*) for 20, 25 and 30 rounds of PCR amplification and probed using the *gag* probe. The below panel shows the DNase-treated viral RNA which was amplified using plasmid specific primer OTR366/367 (hygro primer). +C, plasmid as a positive control. The bottom panel shows Western blot analysis of equivalent amounts of the remaining one fourth purified virus harvested from transfected cultures using the FIV antiserum from infected cats. The relative packaging efficiencies were calculated from ODs of 20 cycles.

Hygro cassette in the background of pcDNA3 (Fig. IV.5, panel A). Semi-quantitative RT-PCR analysis of the transfer vector RNAs packaged in the virions showed that AG021 with the 3' LTR was packaged with a 3-fold enhanced efficiency than AG029 (Fig. IV.5, panel B), confirming the results obtained earlier (Fig. IV.4). Once again, AG021 and AG029 were expressed efficiently in the cytoplasm (Fig. IV.5, panel C), cells were fractionated appropriately (Fig. IV.5, panel D), and similar levels of virions were produced in the transfected cultures (Fig. IV.5, panel B).

The U3 portion of the LTR also contains packaging determinants. To determine whether the increase in the packaging efficiency was due to the presence of the U3 element, we constructed AG051 that expressed the entire LTR, U3/R/U5, from the CMV promoter and compared the packaging efficiency of its RNA with that from AG029, the vector that expressed the R/U5 region only form the CMV promoter (Fig.IV.6, panels A & B). As shown, the addition of U3 increased the packaging efficiency of AG029 by 2 folds, revealing that the U3 region of the LTR also contributes to packaging of the FIV RNA contrary to published findings (Kemler et al., 2004).

The context of the LTR determines its relative contribution to FIV RNA packaging. Since the FIV LTR when present at the 3' end is transcribed until the poly A signal in the R region, we determined whether there was any difference when only U3/partial R sequences were present (up to the poly A signal at nt 253 in the LTR) in the transfer vector RNAs expressed from the CMV promoter (AG053) versus U3/complete R (nt 281) (AG052), or the entire U3/R/U5 (up to nt 357) (AG051) and compared the packaging of these transcripts with those expressed from AG001 (Fig. IV.7, panel A). AG001 expresses the *Hygro* gene from the SV40 promoter using the 3' LTR for transcript

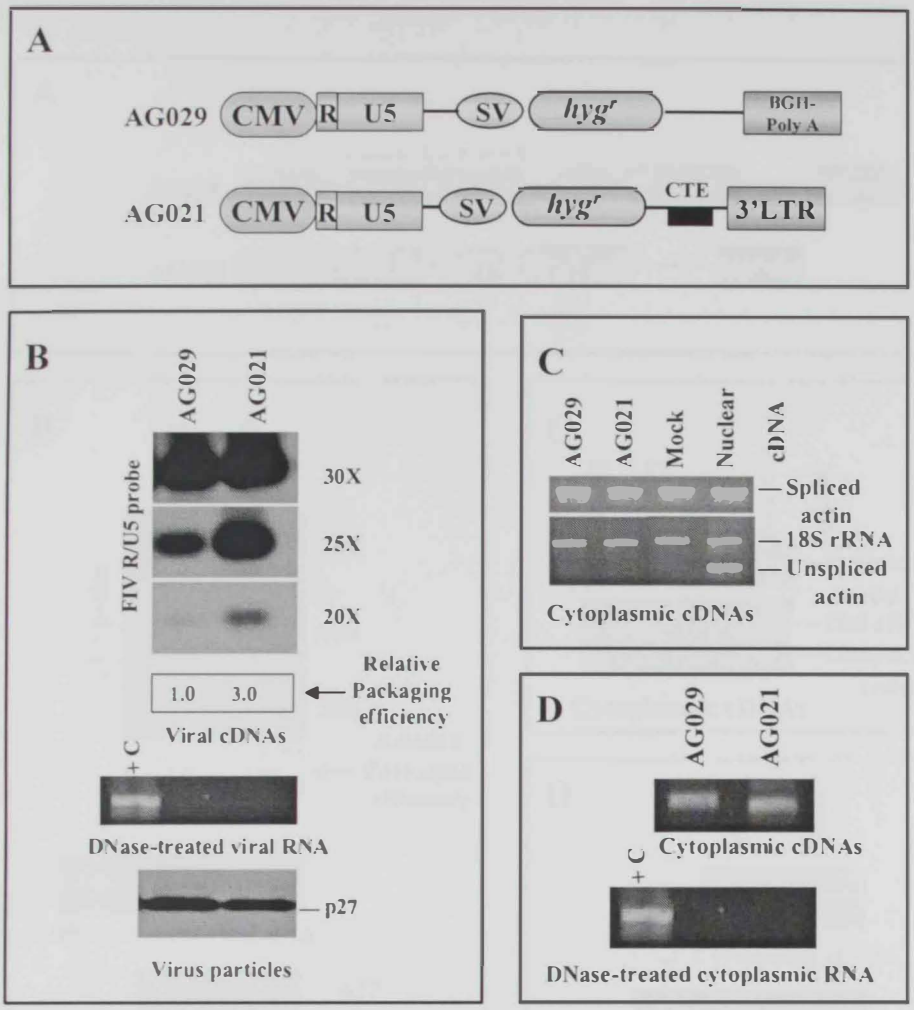

Figure IV.5. The 3' LTR increases the packaging efficiency of FIV RNA. A) Schematic representation of the transfer vectors with and without the 3' LTR. B) Semi-quantitative RT-PCR of viral cDNAs amplified using OTR516 (R) and OTR459 (end of U5) primers for 20, 25 and 30 rounds of PCR and probed using the R/U5 probe. The panel below shows the DNase-treated viral RNA which was amplified using plasmid specific primer OTR366/367 (hygro primer). +C, plasmid as a positive control. The bottom panel shows the Western blot analysis of equivalent amounts of the remaining one fourth purified virus harvested from transfected cultures using the FIV antiserum from infected cats. C) Control for nucleocytoplasmic fractionation technique. The upper panel is the RT-PCR on cytoplasmic RNA for spliced actin which should be present in both the nuclear and cytoplasmic fractions, while the bottom panel is a multiplex RT-PCR for unspliced β-actin mRNA that should be exclusively nuclear and 18S ribosomal RNA as a control for the presence of cDNA. D) RT-PCR of cytoplasmic cDNA amplified using OTR516 (R) and OTR459 (end of U5) primers. The bottom panel shows the DNase-treated cytoplasmic RNA, which was amplified using plasmid specific primer OTR366/367 (hygro primer). The relative packaging efficiencies were calculated from the average of 20 and 25 cycle ODs using different exposures.

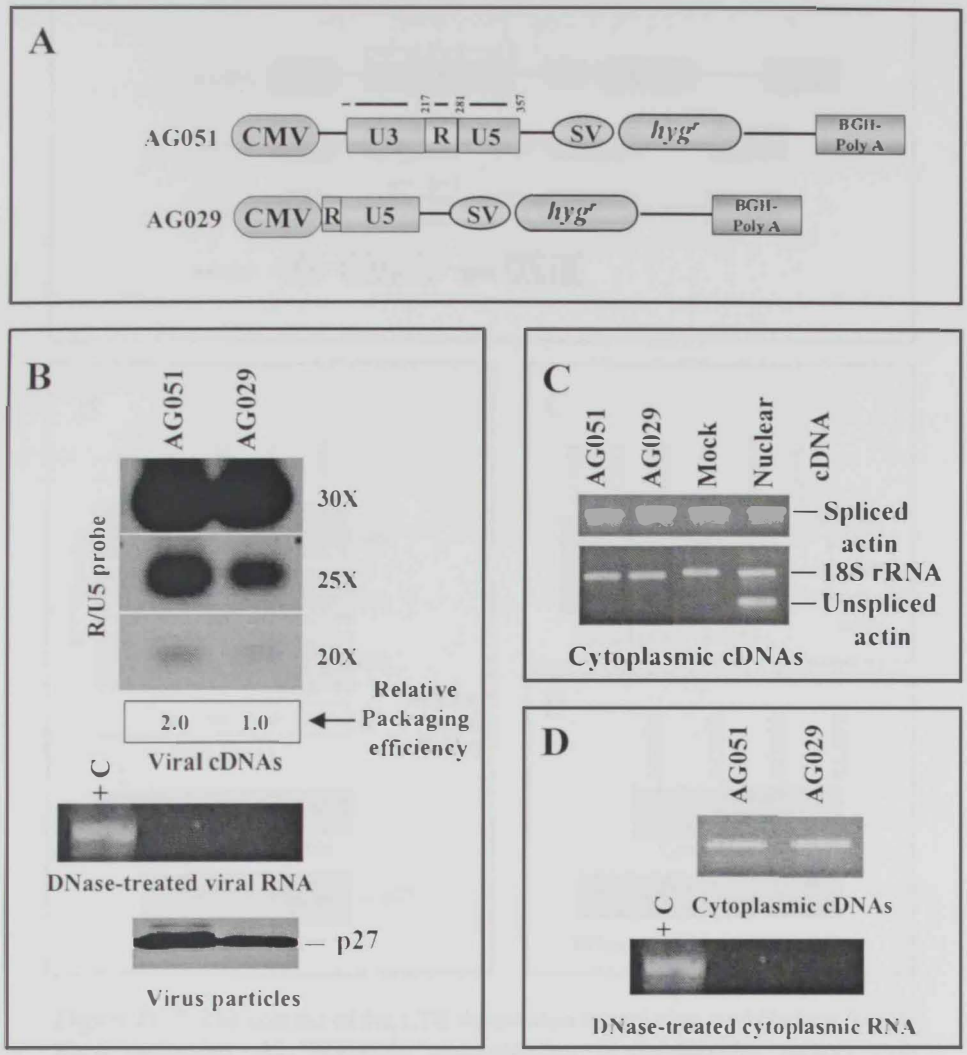

Figure IV.6. The U3 part of the 3' LTR in particular increases the packaging efficiency of FIV RNA. A) Schematic representation of the transfer vectors either with R/U5 or U3/R/U5 in the heterologous context. B) Semi-quantitative RT-PCR of viral cDNAs amplified using OTR516 (R) and OTR459 (end of U5) for 20, 25 and 30 rounds of PCR and probed using the R/U5 probe. The panel below shows the DNase-treated viral RNA which was amplified using plasmid specific primer OTR366/367 (hygro primer). +C, plasmid as a positive control. The bottom panel shows Western blot analysis of equivalent amounts of the remaining one fourth purified virus harvested from transfected cultures using the FIV antiserum from infected cats. C) Control for nucleocytoplasmic fractionation technique. The upper panel is the RT-PCR on cytoplasmic RNA for spliced actin which should be present in both the nuclear and cytoplasmic fractions, while the bottom panel is a multiplex RT-PCR for unspliced β-actin mRNA that should be exclusively nuclear and 18S ribosomal RNA as a control for the presence of cDNA. D) RT-PCR of cytoplasmic cDNAs amplified using OTR660 (R) and OTR601 (SV40). The bottom panel shows the DNase-treated cytoplasmic RNA, which was amplified using plasmid specific primer OTR366/367 (hygro primer). The relative packaging efficiencies were calculated from the average of both 25 and 20 cycles ODs using different exposures.

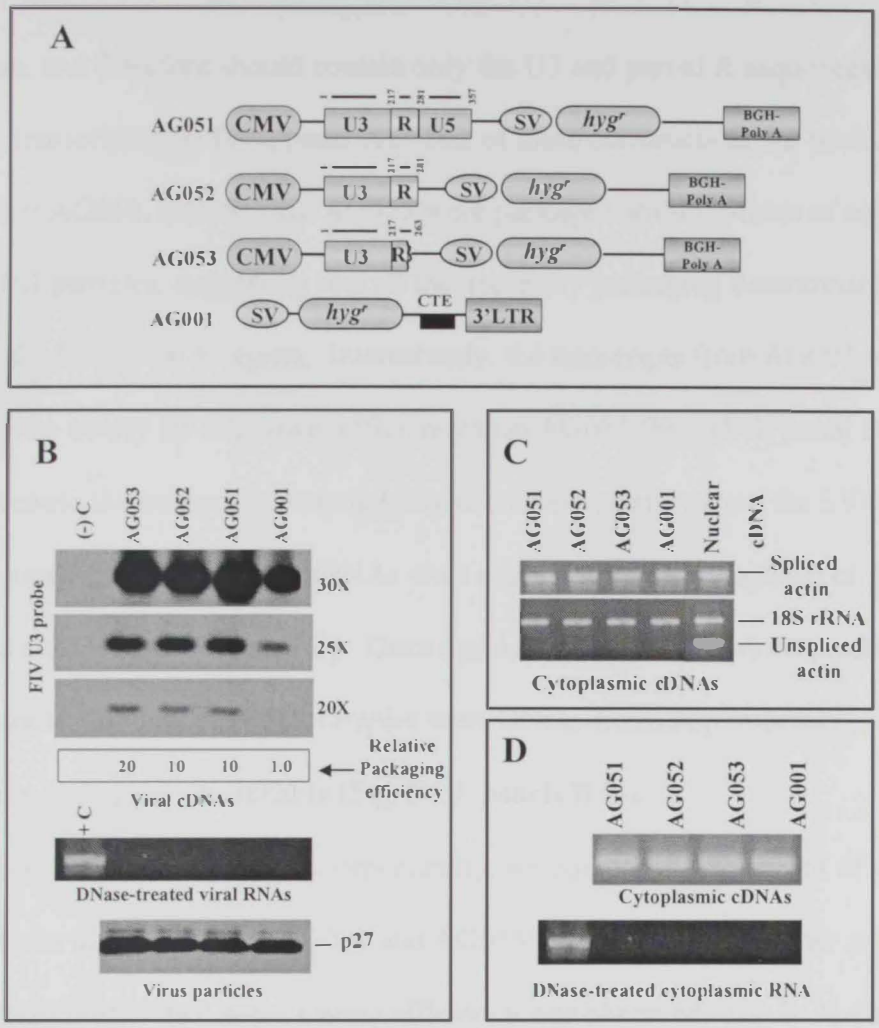

Figure IV.7. The context of the LTR determines the relative contribution to FIV RNA packaging. A) Schematic representation of the transfer vectors with U3/R/U5 or U3/R or U3/partial R, or the 3' LTR in the 3' context. B) Semi-quantitative RT-PCR of viral cDNAs amplified using OTR627 (beginning of U3) and OTR517 (end of U3) primers for 20, 25 and 30 rounds of PCR and probed using the U3 probe. The panel below shows the DNase-treated viral RNA which was amplified using plasmid specific primer OTR366/367 (hygro primer). +C, plasmid as a positive control. The bottom panel shows the Western blot analysis of equivalent amounts of the remaining one fourth purified virus harvested from transfected cultures using the FIV antiserum from infected cats. C) Control for nucleocytoplasmic fractionation technique. The upper panel is the RT-PCR on cytoplasmic RNA for spliced actin which should be present in both the nuclear and cytoplasmic fractions, while the bottom panel is a multiplex RT-PCR for unspliced β-actin mRNA that should be exclusively nuclear and 18S ribosomal RNA as a control for the presence of cDNA. D) RT-PCR of cytoplasmic cDNAs amplified using OTR627 (beginning of U3) and OTR517 (end of U3) primers. The bottom panel shows the DNase-treated cytoplasmic RNA, which was amplified using plasmid specific primer OTR366/367 (hygro primer). The relative packaging efficiencies were calculated from the average of 20 and 25 cycle ODs using different exposures.

termination, and therefore should contain only the U3 and partial R sequences at the 3' end of the transcript (Fig. IV.7, panel A). Test of these constructs in our packaging assay revealed that AG051, AG052, and AG053 were packaged within 2-folds of each other in the FIV viral particles, suggesting that all the necessary packaging determinants were present in the U3/partial R region. Interestingly, the transcripts from AG001 were packaged with nearly 10-fold lower efficiency than AG053 (Fig. IV.7, panel B). This was not because the transcripts were not produced appropriately from the SV40 promoter in AG001 since similar levels of mRNAs were expressed in the cytoplasm of the transfected cells (Fig. IV.7, panel D). Once again, the amount of virions produced were similar in these cultures, the RNA samples were DNase-treated appropriately, and the fractionation technique was reliable (Fig. IV.7, panels B-D).

To confirm these findings independently, we compared the amount of virion RNA packaged from transfer vectors AG001 and AG053 using a different primer pair. Once again, a 20-fold difference in packaging efficiency was observed (Fig. IV.8, panel A). Additionally, we compared the amount of virion RNA packaged from transfer vectors AG001 with AG021. AG021 is similar to AG001 in that it uses the 3' LTR for transcript termination, but uses the CMV promoter to express the RNA, thus by-passing the need for the SV promoter. Test of these constructs revealed that both vectors were packaged within 3-folds of each other, an effect attributed to the presence of R/U5 sequences in AG021 (Fig. IV.8, panel B). These results clearly show that the FIV LTR when expressed in the 3' context is less efficient in enhancing packaging than when present at the 5' end.

The U3/R folds differently when at the 3' end versus when at the 5' end. To determine why the packaging efficiency of U3/R determinants differed when expressed from the 5'

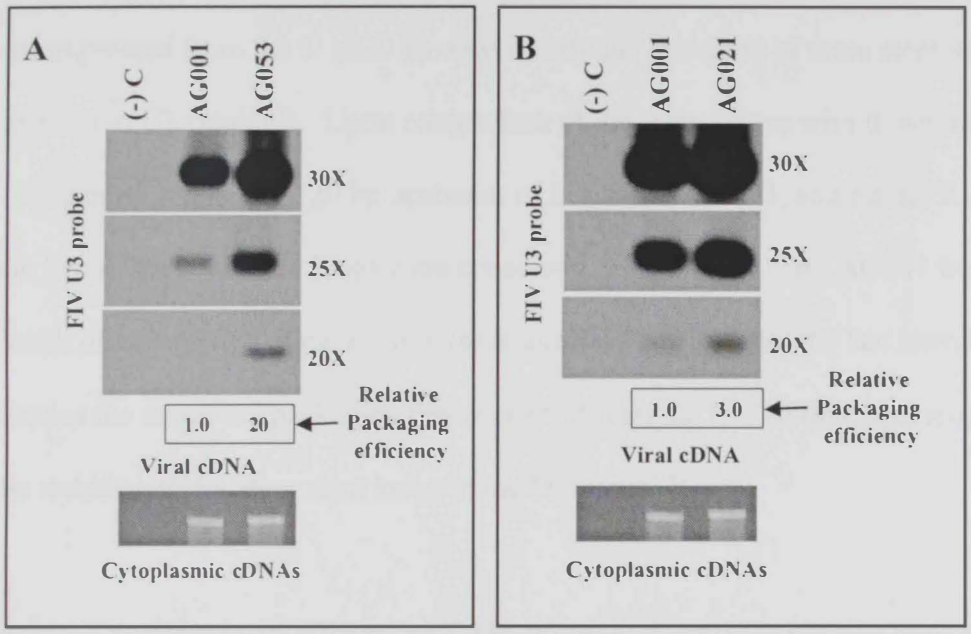

Figure IV.8. The 5' context of the LTR has better packaging potential than the 3' context. A) Semi-quantitative RT-PCR of viral cDNAs amplified using OTR627 (beginning of U3) and OTR517 (end of U3) primers for 20, 25 and 30 rounds of PCR and probed using the U3 probe. B) Semi-quantitative RT-PCR of viral cDNAs amplified using OTR627 (beginning of U3) and OTR517 (end of U3) primers for 20, 25 and 30 rounds of PCR and probed using the U3 probe. The relative packaging efficiencies were calculated from the ODs obtained from 25 and 20 cycles.

versus the 3' end, the U3/R/U5 was folded in the 5' and 3' contexts. At the 5' end, these sequences were expressed from the CMV promoter, while at the 3' end, the FIV LTR was cloned along with ~120 bp of the upstream adjacent FIV sequences that contain coding sequences for the 3' end of *env* and *rev* exon 2. As shown in Fig. IV.9, the U3/R/U5 folded into four stem loop structures that were conserved whether U3/R/U5 or U3/R were expressed from the 5' end. However, only the last three of these stem loops were conserved in U3/partial R. Upon comparison of these structures with those from AG001 (containing sequences 120 bp upstream of 3' LTR, entire U3, and partial R), only the middle two of the four stem loops were conserved. Since RNA from AG001 was the least efficient of all in terms of packaging, these analyses suggest that the last stem loop either contains the major U3 packaging determinant or that the loss of this stem loop affects the stability of the other stem loops in the U3/partial R.

The packaging determinants within the UTR and *gag* constitute the core packaging determinants of FIV.

Finally, using our already constructed vectors, we asked the question as to which of the regions of FIV contains the primary packaging determinant, R/U5 at the 5' end, 150 bp UTR/100 bp *gag*, or the 3' LTR. Since we were limited by the primer pairs we could use for a simultaneous analysis of the constructs, vectors were chosen that contained R/U5 in common (Fig. IV.10, panel A). Semi-quantitative RT-PCR analysis of these constructs revealed that R/U5 on its own was a weak packaging signal. The packaging efficiency increased by ~ 4-folds in the presence of the 3' LTR, as observed before. With the addition of the 90 bp UTR/100 bp *gag*, the packaging efficiency increased further and

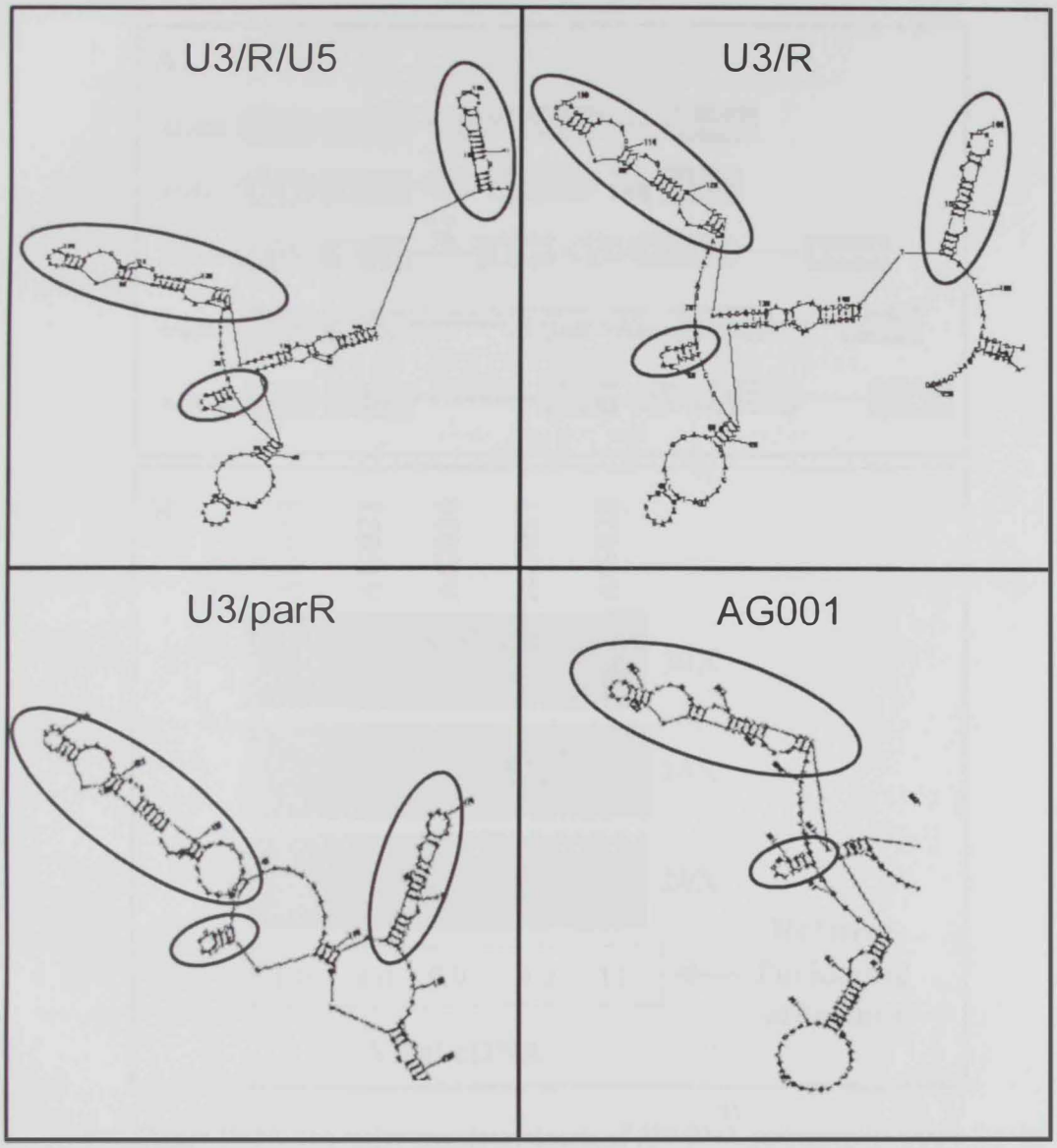

Figure IV.9. Folding potential of the FIV transfer vector RNAs expressing the U3, R, or U5 elements in either the 5' or 3' context. The ovals represent the conserved stem loop structures.

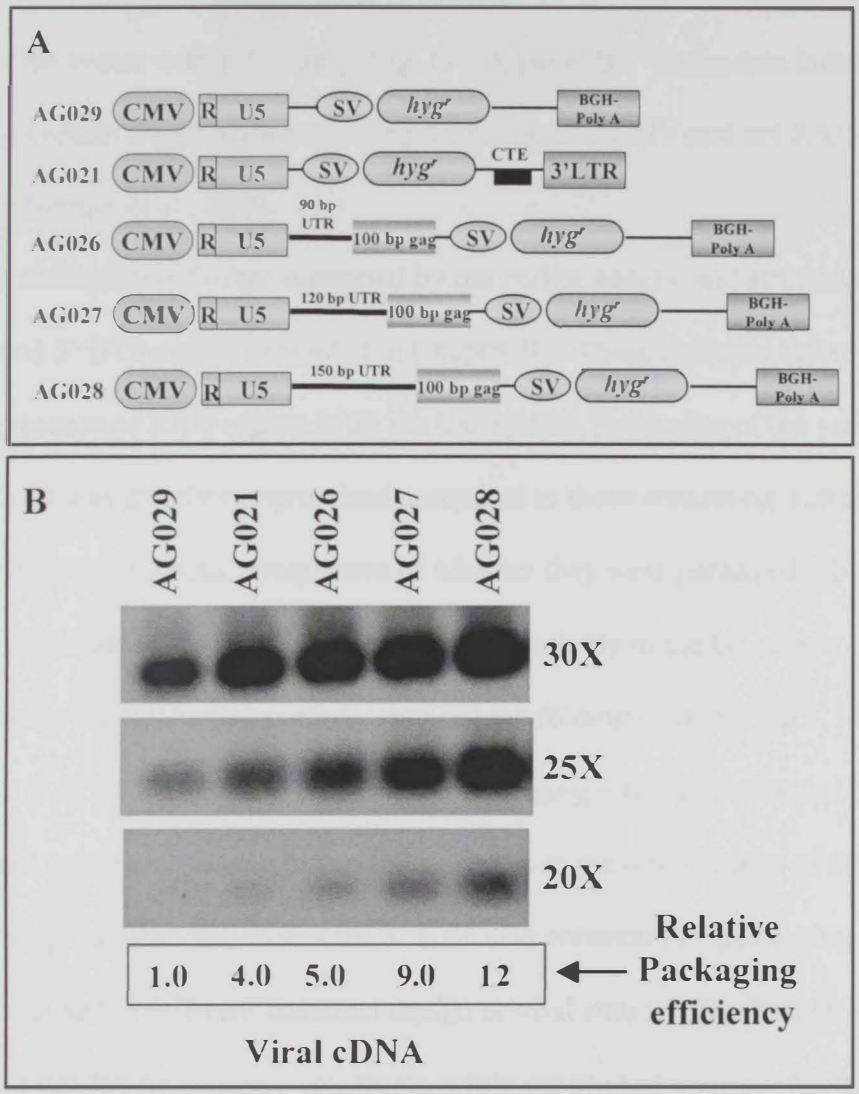

Figure IV.10. The major core determinants of FIV RNA packaging lie within 150 bp of the 5' UTR and 100 bp of *gag* while R/U5 and U3 play a minor role in FIV RNA packaging. A) Schematic representation of the vectors tested. B) Semi-quantitative RT-PCR of viral cDNAs amplified using OTR660 (R) and OTR459 (end of U5) primers for 20, 25 and 30 rounds of PCR and probed using the R/U5 probe. The relative packaging efficiencies were calculated from the ODs of 20 cycles.

each incremental increase in the UTR kept increasing the packaging efficiency till 150 bp were reached when maximum packaging efficiency was observed, ~ 12-fold over that observed for the vector with R/U5 only (Fig. IV.10, panel B). These data indicate that the UTR/*gag* contain the primary packaging determinants of FIV and not R/U5, as suggested by Kemler et al., 2002.

This conclusion is further supported by our earlier genetic and structural analysis of the LTR and 5' UTR region presented in Chapter III. These analyses had revealed that despite the presence of R/U5 region in all the test vectors, packaging of the vector RNAs with 90 bp UTR was greatly compromised compared to those containing 120 and 150 bp UTR. All these vector RNAs, irrespective of whether they were packaged efficiently or inefficiently, maintained the stem loop structure, SL1, stably in the U5 region of the RNA, while only the RNAs that partially restored the folding of stem loop 2, SL2, present in the 5' UTR were able to enhance the packaging efficiency of their RNAs.

Several reasons may explain the discrepancies in the observations of the two studies including our observation that the 3' LTR also contains FIV packaging determinants related to different construct design or viral strains. The Kemler study, for example, used full-length genomic constructs, while our studies were conducted using non-viral heterologous or subgenomic constructs that lacked most of the sequences encoding for the structural genes. Presence of negative inhibitory elements within structural genes such as *gag*, *pol*, and *env* have been shown to affect expression of unspliced mRNAs (transport, stability, and translatability) and hence could theoretically affect the subsequent incorporation of the unspliced mRNA into virions (reviewed in Cullen, 2003). Vector design itself can sometimes pose problems such as those observed

by Poeschla and colleagues who reported that addition of *gag* sequences beyond nt 311 negatively impacted the ability of such transfer vector RNAs to be packaged into virions possibly related to the presence of the highly structured RRE in their vectors (Poeschla et al., 1998a). Finally, it seems that Kemler et al. (2002) used the Petaluma strain for their analysis which is what we used as well; however, their UTR is about 273 bp in length compared to 270 bp in our case, suggesting that perhaps a different molecular clone of FIV Petaluma was analyzed by them.

Together with the results presented in Chapter III, our studies reveal that FIV RNA packaging determinants are as complex as those of other lentiviruses, reflecting the multidimensional nature of how genomic RNAs are packaged by the assembling virion. This requires not only recognition of the RNA structural features by the Gag polyproteins to distinguish between sub-genomic and genomic mRNAs, but also issues related to RNA dimerization. Sequences responsible for retroviral RNA dimerization co-map to regions similar to RNA packaging determinants and despite intensive investigations, it is still not clear whether RNA packaging precedes retroviral RNA dimerization or comes later (Russell et al., 2004).

Chapter V

CONCLUSIONS & RECOMMENDATIONS

This thesis was undertaken to fine map the sequences required for efficient FIV RNA packaging into the viral particles. Using our previously published results, we embarked upon a two-pronged strategy to address this question. Our previous results revealed that the FIV packaging determinants consisted of two core elements, one encompassing ~120 bp of 5' UTR while the other about the first 100 bp of *gag* (Browning et al., 2003 a & b). To fine tune our mapping, we conducted an incremental deletion analysis of the intervening sequences between the two core elements, or substituted the deleted region with heterologous sequences of same length to determine whether the intervening region itself played a role in FIV RNA packaging, or simply acted to maintain the distance between the two core elements (Chapter III).

We went on to dissect the role of the LTR elements towards packaging since it was suggested that LTR elements may have a key role to play in packaging, but only when present at the 5' end of the viral RNA (Kemler et al., 2002). Since our earlier constructs maintained most of the 5' LTR and all of the 3' LTR, we constructed another series of vectors that tested the effects of the U3, R, and U5 sequences on FIV RNA packaging in both the 5' and 3' contexts (Chapter IV).

Our incremental deletion analysis of the region intervening the two core packaging determinants confirmed the earlier observations that the major determinants of FIV RNA packaging lie within two discontinuous regions, the first 150 bp of 5' UTR and the first 100 bp of *gag* (Chapter III). Furthermore, we demonstrated that the sequences in between the two core packaging determinants are dispensable for packaging; neither do they affect packaging directly, nor do they act as spacers to maintain any structural features of the region (Chapter III). In terms of the LTR elements, our data revealed that

both the 5' R/U5 region and the 3' LTR contribute to FIV RNA packaging and to a similar extent (Chapter IV). The U3 region was also shown to affect packaging specifically (Chapter IV). A comparative analysis of all the regions involved in packaging revealed that the first 150 bp UTR in combination with the first 100 bp of *gag* had the strongest influence on packaging, making them the core packaging determinants (Chapter IV).

Since retroviral RNA packaging determinants are thought to fold into higher order structures, we folded the RNA regions found to be important for packaging using the RNA folding algorithm, RNAstructure (Mathews et al., 1999). Analysis of the predicted structures revealed that the 5' end of the FIV genomic RNA folded into several stable stem loops, a situation similar to HIV, SIV and MPMV (Fig. V.1 & Fig. I.7). Two stem loop structures were observed in the R/U5 region (labelled "R" and "R/U5" in Fig. V.1), but only transiently, suggesting that more than likely they are not involved in RNA packaging since they are unstable to begin with. Similarly, a structure with a huge loop was identified within the first 100 bp of *gag* (labeled "AUG Gag"), but also not consistently, suggesting that the *gag* region probably does not function at a structural level either (see Chapter III). In between these unstable structures, we observed four stable stem loops designated SL1-4 (Fig. V.1). SL1 was found within U5, a region involved in packaging; however, we feel that this stem loop is not significantly involved in packaging since it was present in vector RNAs that were packaged poorly (AG002/AG013, Chapter III). SL2, found within the first ~180 bp of the UTR, was the most consistently formed stem loop structure found to be associated with enhanced packaging (Chapter III). Even the emergence of the first two loops of SL2 were found to

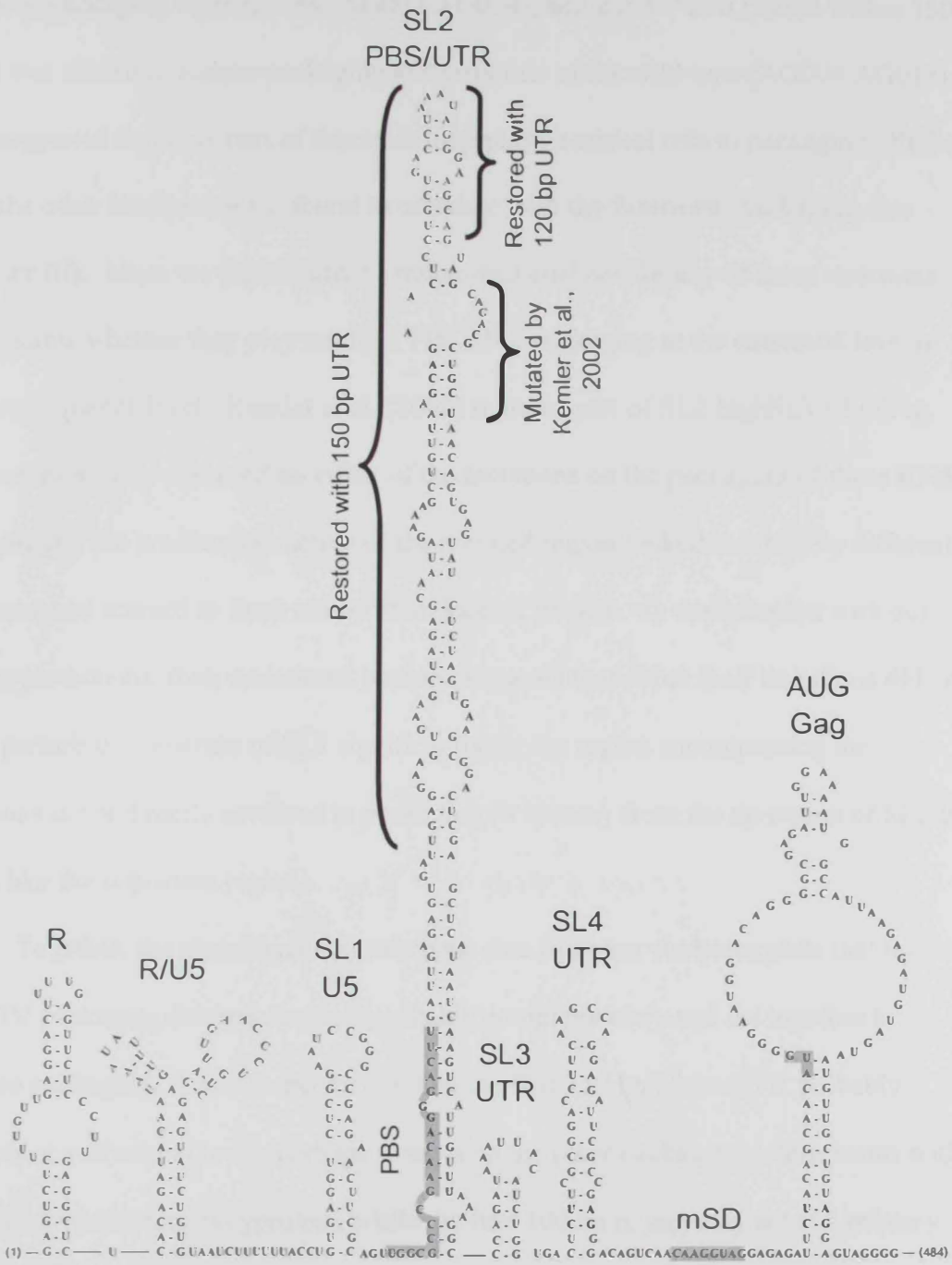

Figure V.1. The predicted secondary RNA structure of the 5' end of the FIV RNA from the cap site (nt 1) to the first 74 bp of *gag* (nt 484). The major *cis*-acting sequences of the virus are highlighted by the gray shading such as the primer binding site (PBS), major splice donor (mSD), and the *gag* initiation codon. The structural features are labeled according to the region of the viral genome they exist in. The regions of stem loop (SL) 2 reformed in the presence of 120 and 150 bp UTR are shown by the brackets along the sides along with the mutations in SL2 introduced by Kemler et al., 2002.

improve packaging significantly (AG003/AG014), while 2/3^{rds} of it (found within 150 bp UTR) was shown to restore packaging to nearly that of the wild type (AG004/AG015). This suggested that only part of this stem loop plays a critical role in packaging. SL3 and 4, on the other hand, were not found to correlate with the functional packaging data (Chapter III). Since we did not carry a mutational analysis for any of these structures, we do not know whether they play a role in FIV RNA packaging at the structural level or at a primary sequence level. Kemler et al. (2002) mutated part of SL2 highlighted in Fig. V.1; however, they observed no effect of the mutations on the packaging of these RNAs. Interestingly, the predicted structure of the mutated region looked completely different from ours and seemed to form a short stem loop of its own. In combination with our folding predictions, their mutational analysis suggests that either their mutations did not really perturb the structure of SL2 significantly, or the region encompassing the mutations is not directly involved in packaging (it is away from the tip region of SL2 that seems like the important region).

Together, the mutational and structural data from our study suggests that the two core FIV packaging determinants are probably complementary and act together to enhance packaging. It is our speculation that the first 150 bp of the UTR probably interacts at a structural level (perhaps along with the other packaging determinants within the LTR) with the Gag polyprotein, while the first 100 bp in *gag* may act at a primary level and be involved more in the identification of the genomic mRNA from the multitude of subgenomic mRNAs that would lack this intronic region. This emerging scenario seems opposite to what we have observed in MPMV in terms of the contribution of the two elements to packaging (Schmidt et al., 2003; Mustafa et al., 2004). Deletion

and site directed mutagenesis of the first four stem loops of the MPMV 5' UTR region has identified sequences encompassing SL1 as the core determinant of MPMV RNA packaging, but at the primary sequence level, while the first ~130 bp of *gag* seem to fold into 3 short stem loops that may be responsible for providing the structural interaction(s) needed for specific incorporation of the genomic RNA into the virus particle. The requirement of the *gag* stem loops to efficient packaging remains to be genetically confirmed.

The region between the 5' mSD and the start of the *gag* (which is around 20 bp) is not critical for FIV RNA packaging since a deletion in this region had minimal effects on the packaging efficiency. (Browning et al., 2003a ; Kemler et al., 2002). However, in the absence of *gag*, we have shown that this region becomes critical for FIV RNA packaging since deletion of this region results in the inability of the core packaging determinant present within the 150 bp of the 5' UTR to function as a packaging signal (Browning et al., 2003a). Thus, despite the absence of core packaging sequences in between the SD and Gag ATG, FIV RNAs lacking this region are packaged highly inefficiently in the absence of *gag*, further suggesting that *gag* sequences have a stabilizing role on the packaging potential of FIV vector RNAs.

The involvement of *gag* sequences as part of packaging determinants of viruses where the core determinant seems to be upstream of the mSD (and hence part of all mRNAs) provides a convenient way of differentiating between spliced and unspliced mRNAs. This may be true not only for FIV, but also for SIV and MPMV (Strappe at al., 2003; Schmidt et al., 2003). However, in the case of HIV-2 which has its packaging determinant upstream of the mSD also, there does not seem to be any *gag* sequence

involvement at the packaging signal level. HIV-2 has solved the problem of genomic mRNA recognition by using a unique sorting mechanism where only those mRNAs capable of translating Gag in *cis* can be packaged into the virus particle. These mRNAs are captured by the co-translated Gag polyproteins while being translated on polysomes in the cytoplasm (Kaye and Lever, 1999). Although no direct evidence is presented in this thesis, one may speculate that *gag* may be involved in a similar co-translational mechanism for FIV (and the same may be true for other retroviruses with their core packaging determinants upstream of the mSD such as SIV and MPMV (Strappe et al., 2003; Schmidt et al., 2003)).

As noted earlier, Strappe et al. (2003) have proposed that it is not the position of the mSD relative to the core packaging determinant that may be important for specific encapsidation of genomic RNAs, rather the distance between the cap site and the core packaging determinants as it may affect the competition between translation and encapsidation that the genomic RNAs must undergo. The distance between the core packaging signal relative to the cap is 300-400 bp in SIV, HIV-1, and HIV-2. In comparison, this distance seems to be much shorter in FIV with only ~150 bp in between the cap site and the start of the core packaging determinant. The significance of this observation is not clear. Perhaps, in viruses where *gag* sequences are also part of the core packaging determinant such as FIV, this distance may not be that critical for genomic RNA recognition. A similar observation has been made in MPMV where the distance between the cap site and the primary packaging determinant is only ~140 bp as well. The core packaging determinant of MPMV resides within the first 30 bp downstream of the PBS, but also requires the first ~100 bp of *gag* sequences and the last 23 bp of the 5'

UTR for efficient RNA packaging (Schmidt et al., 2003; Mustafa et al., 2004).

In short, our results reveal that similar to other complex lentiviruses, the FIV packaging signal is complex and multipartite, residing primarily within the first 150 bp of the 5' UTR and the first 100 bp of *gag*. However, other determinants of packaging also seem to be present in the R/U5 region of the 5' LTR and the U3/R region of the 3' LTR. Since FIV-based self-inactivating vectors (SIN) would be good candidates for human gene therapy, the dispersal of FIV RNA packaging determinants makes this task slightly tricky, especially the involvement of the R element in the 3' LTR. Several strategies are available for making SIN vectors that exploit steps of reverse transcription to prevent generation of packageable vector RNAs. These include the use of repeat sequences or splice sites to remove the packaging signal during reverse transcription of the vector RNA (summarized in Browning, 2001). We will have to use a combination of such strategies to develop effective FIV-based SIN vectors, including deletion of not only the core packaging determinants in the 5' UTR and *gag*, but also deletion of as much of the U3 region of 3' LTR as possible (permissible since the marker/therapeutic gene will use an internal promoter and will not need the viral promoter for expression). The fact that we could delete major portions of the 5' UTR without affecting vector RNA propagation significantly (see Table III.1) suggests that such deletions should not be detrimental to the ability of the vector RNA to complete the reverse transcription and integration process. Our prediction is that the remaining packaging determinants within the R region in the 3' LTR in the presence of minimal U3 sequences should not be able to function as an effective packaging signal, or will have highly reduced packaging potential, resulting in the minimization of the packaging potential of the FIV transfer vector RNAs.

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على الدمج من خلال تجربة على الخلايا الحية. وعلى إثر ذلك تم تحليل كميات من (RNA) التي تم دمجها مباشرة في جزيئات الفيروس باستخدام نظام (RT-PCR).

أكدت التحاليل ما ذهبنا إليه من أن عوامل دمج فيروس نقص المناعة لدى القطط (FIV) هي بالفعل غير متصلة وتعطي عوامل الدمج الرئيسية والتي تمتد من ١٥٠ نكليوتيد (UTR) و ١٠٠ نكليوتيد (Gag). بالإضافة إلى ذلك فإن السلاسل التي تتوسط هذين العنصرين ليست ضرورية لعملية دمج أو انتشار المادة الوراثية. وأدى تحليل عناصر (LTR) إلى اكتشاف عوامل دمج أخري أقل فاعلية من السابقة وتقع في مناطق (5'R/U5) و (3'U3/R) ضمن (LTR). لقد تبين من تشكيل منطقة (5'end) الخاصة بالـ (RNA) الفيروسي عن طريق الكمبيوتر وجود تركيبات معقدة ومتكونة من جذوع وحلقات. وعند ربط عملية التحول بعمليات التنشئة اتضح بقاء تركيبة واحدة مكونة من جذع وحلقة داخل (5'UTR) يمكن اعتبارها العامل الرئيسي في عملية دمج فيروس نقص المناعة لدى القطط (FIV). وجدير بالذكر هنا أنه لم يوجد أي تركيبة على امتداد المائة نكليوتيد الأولى في (Gag) يمكن اعتبارها مسؤولة عن دمج الفيروس في منطقة (Gag)، لذلك يمكن اعتبار تتابعات (Gag) فعالة فقط خلال المراحل الأولى كأن تولد تتابعات داخلية يمكن عن طريقها التفرقة بين (mRNA) المتقطعة والغير متقطعة. وستزودنا كل هذه المعطيات بالمعارف الضرورية لفهم عملية دمج الفيروسات الارتجاعية المعقدة للمادة الوراثية (RNA) وللمساعدة على تصميم ناقل للفيروس يمتاز بالقدرة على كبح نفسه ويساعد في العلاج الجيني للإنسان.

الملخص العربي

إن عملية دمج الفيروسات الارتجاعية (Retroviruses) لمادتها الوراثية يمثل إحدى الوظائف الدقيقة والتي يتمكن بها الفيروس من دمج السلاسل الغير متقطعة الخاصة به من ضمن ملايين من سلاسل (mRNA) الفيروسية والخلوية المتقطعة الموجودة في السيتوبلازم. وتتطلب عملية الانتقاء هذه أن يحتوي الحمض النووي الريبي (RNA) الوراثي على عناصر دمج خاصة به والتي تتفاعل بأسلوب معين مع البروتينات الدامجة الخاصة بالجزيئات الفيروسية أي البروتينات المتعددة لعنصر (Gag). إن تحديد السلاسل المسؤولة عن عملية الدمج سيمكننا من تحديد عملية الدمج الخاصة بالحمض النووي الريبي (RNA) الوراثي المتكامل.

لقد ارتأينا تحديد العوامل المساعدة على عملية الدمج الخاصة بفيروس نقص المناعة لدى القطط (FIV) وهو فيروس يصيب الخلايا الغير منقسمة ويعتبر من الأنظمة الناقلة الفعالة في إطار العلاج الجيني للإنسان. لقد توصلنا من خلال أبحاثنا الأولية إلى أن العوامل المساعدة على دمج فيروس نقص المناعة لدى القطط (FIV) تتمركز في منقطتين منفصلتين أساسيتين ضمن منطقة (5'end) من المادة الوراثية الفيروسية. وتمتد المنطقة الأولى من (R/U5) بمنطقة (5'LTR) إلى غاية ١٢٠ نكليوتيد بمنطقة (5'UTR)، بينما تغطي المنطقة الثانية المائة نكليوتيد الأولى من جين ال (Gag)، إلا أنه يمكن لمناطق أخرى من الجينوم أن يكون لها دور أيضا.

تناولت هذه الأطروحة تلك المعطيات بمزيد من الدرس والتعمق من أجل معرفة ما إذا كان للمنطقة الوسطى دور في عملية الدمج أم أنها مجرد منطقة فاصلة بين العنصرين الأساسين في تلك العملية. بالإضافة إلى ذلك فإنه قد تم التوصل في دراسات أخرى إلى أن عدة مناطق في الجينوم وخاصة (LTR) تحتوي على عناصر دمج هامة، ولذلك فقد فصلنا دور عناصر (LTR) عن منطقة (UTR) أثناء قيامنا بدمج المادة الوراثية لفيروس نقص المناعة لدى القطط (FIV). ولتحقيق ذلك تم تكوين مجموعات لنقل فيروس نقص المناعة لدى القطط (FIV) في بيئة تحتوي على خليط من (5'FIV UTR) و (Gag) وتم قياس قدرتها

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