Construction of a Retroviral Vector for Production of Transgenic Cells Expressing Feline Immunodeficiency Virus (FIV) Viral Protein

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ABSTRACT

Retroviral vectors are being used as vehicles for transporting foreign genes into mammalian cells for the purpose of human gene therapy to treat viral disease like human immunodeficiency virus (HIV). In efforts to develop a preventative vaccine against a lentiviral infection, research is centering on developing a protective vaccine that will identify antigens which induce protective cell mediated immunity, especially cytotoxic T lymphocytes (CTL). Retroviral vectors provide the required endogenous expression of a retrovirus antigen for identificatoin of CTL responses against that individual antigen. Feline immunodeficiency virus (FIV) is an animal lentivirus that shares common clinical properties with HIV. The commonalities shared between HIV and FIV allows the use of FIV as an animal model for developing a protective vaccine that will identify antigens which induce protective cell mediated immunity or CTL responses. The FIV *gag* nucleocapsid (p10) protein will be cloned into the retroviral vector pLXSN for production of transgenic cells expressing the p10 protein for study of specific T-cell responses to this FIV antigen.

PCR will be used to amplify the nucleocapsid from the pUC119 plasmid containing the FIV genome. The amplified p10 PCR product will be EcoRI and BamHI digested for directional insertion into the EcoRI and BamHI digested retroviral vector pLXSN. Transformation of competent E. coli cells will provide colonies containing the recombinant retroviral vector DNA. Analysis of the recombinant retroviral vector DNA will be carried out by PCR.

Amplification by PCR of the nucleocapsid protein provided the p10 DNA sequence. Subsequent EcoRI and BamHI digestion revealed a natural EcoRI site at base pair 1871, therefore cutting off 160 bp from the p10 sequence. The upstream primer was reconstructed with an XhoI cut site for reamplification of the p10 protein from the pUC119 plasmid and will be inserted into the retroviral vector by XhoI and BamHI digestion. The 204 bp sequence generated from the EcoRI and BamHI digestion was inserted into the retroviral vector and awaits analysis of the idetification of recombinant retroviral vector colonies.

INTRODUCTION

Retroviruses

Retroviruses are an RNA virus that propagates via conversion into duplex DNA. They were first discovered in 1904 with the identification of equine infectious anemia virus (EIAV) (Vallee and Carre, 1904). Many different retroviruses have since been isolated and classified into the Retroviridae family. Retroviridae is currently divided into three subfamilies: the oncovirinae, lentivirinae and spumavirinae. Oncovirinae is the largest group of retroviruses and categorizes those viruses with the ability to transform cells and cause tumors. The lentivirinae subfamily is characterized by the ability of the virus to induce immunodeficiency in a host with a long latency period. Human immunodeficiency virus (HIV) and feline immunodeficiency virus (FIV) are two examples of this subfamily. The last subfamily, spumavirinae, incorporates those viruses that induce vacuoles in cultured cells and are not known to cause disease, but are thought to act as co-factors for other viral diseases (Li, 1995).

A retrovirus is composed of a genome that is single stranded RNA wrapped in a core of viral proteins which, in turn, is enclosed in an envelope made of viral glycoproteins and host cell membrane (Figure 1). For replication, the retrovirus generally uses the host cell's machinery, however it carries its own enzymes and RNA for synthesis of the double stranded DNA from its RNA genome and splicing of that DNA into the host chromosome (Benedict, 1994).

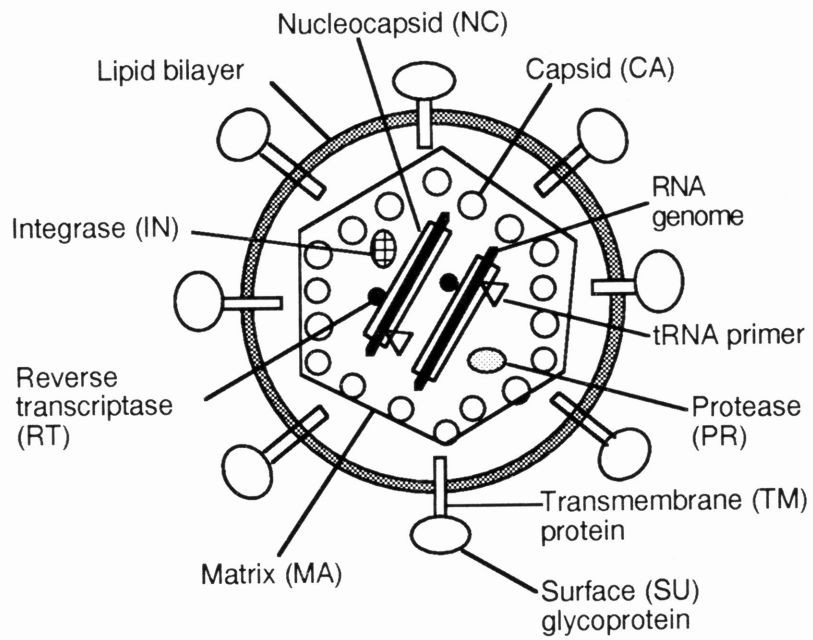The retroviral genome is a complex of two identical, single

Figure 1. Retrovirus model.

stranded RNAs. The RNA sequence, R-U5-PBS-Ψ-gag-pol-env-PPT-U3-R, can be functionally divided into two parts (Figure 2). Part one consists of the cis-acting elements that control viral replication. These sequences - U3, U5, R, PBS (primer binding site), PPT (polypurine tract) and Ψ - include signals for initiation and progression of DNA synthesis, for integration, for transcription of the proviral DNA into RNA, for RNA processing, and for packaging RNA into progeny virions. The second part consists of sequences called ORF or open reading frames, that encode the viral structural proteins (encoded by gag and env), enzymes (encoded by pol), and proteins with regulative functions (encoded by oncogenes, rev, tat, etc.). These sequences are trans-acting and can be deleted without affecting the production of infectious viral particles as long as the deleted viral proteins are provided in trans by another source.

Among the different strains of retroviruses, the sequence of the gag gene is relatively conserved (Doolittle *et al.*, 1990). The gag gene encodes a polyprotein which is post-translationally cleaved into the matrix protein (MA), the capsid protein (CA), and the nucleocapsid protein (NC). The nucleocapsid protein binds to the RNA genome and is thought to be essential for packaging the genomic RNA into the virion (Gorelick *et al.*, 1988; Meric *et al.*, 1986). The capsid protein is involved in the formation of the viral core which surrounds the viral genome. The matrix protein is modified at the amino terminus by the addition of the fatty acid myristic, which is consistent with the belief that this protein is membrane associated and is important for acquisition of the envelope.
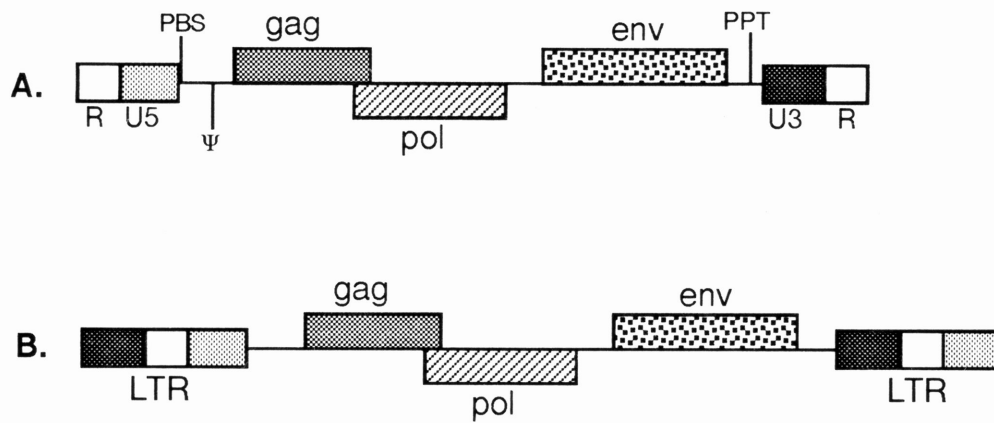
Figure 2. Genomic structure of a retrovirus. A. viral genomic RNA. B. proviral DNA.

The enzymes responsible for converting the retrovirus RNA genome into DNA are encoded by the pol ORF. These include: a protease that is responsible for the cleavage of gag and pol polyprotein; a reverse transcriptase (RT) that is an RNA-dependent DNA polymerase responsible for synthesizing the double stranded DNA from a single stranded RNA template in the reverse transcription reaction; and an integrase (IN) that cuts DNA and rejoins the inserted DNA to the target DNA sequence (Temin and Mizutani, 1970; Baltimore, 1970; Brown *et al.*, 1989). Reverse transcriptase and integrase are involved in the early stages of viral infection, in that they are responsible for converting the single stranded RNA genome into the double stranded DNA intermediate and inserting the DNA provirus into the host cell chromosome.

The env ORF of a retroviral genome encodes the viral envelope polyprotein which is later cleaved to produce glycosylated surface (SU) and transmembrane (TM) proteins. The surface protein is the larger of the two proteins and is involved in binding of the retrovirus to the receptor on the cell surface. The transmembrane protein is responsible for anchoring both the SU and TM proteins to a viral envelope and in fusing of the envelope with a cell membrane to facilitate entry of the virus into the cell (Li, 1995).

The life cycle of a retrovirus begins when the viral surface proteins attach to a receptor on the cell surface (Figure 3). Fusion of the viral envelope and the cellular membrane occur, followed by internalization of the virion and the conversion of the single stranded RNA viral genome into double stranded DNA. The newly synthesized DNA viral strands are transported to the nucleus
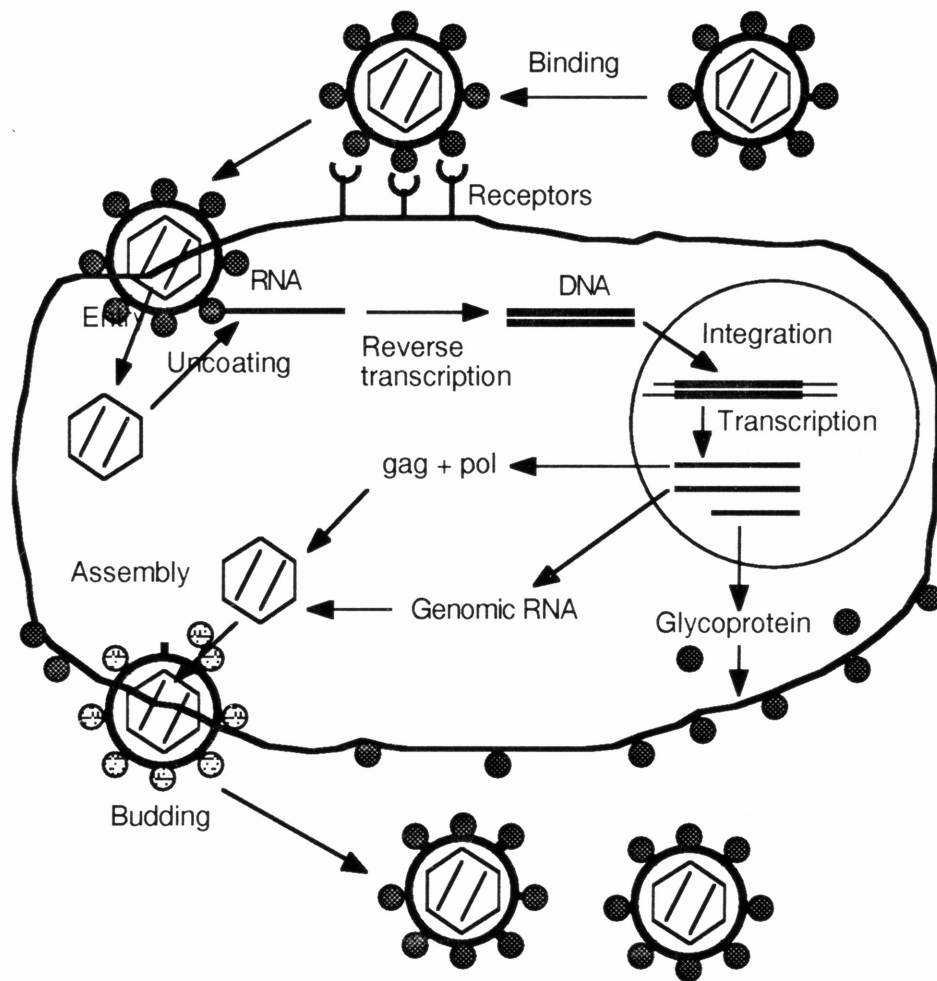
Figure 3. Life cycle of a retrovirus.

of the cell and integrated into the host cell chromosome to become a provirus. The virus then uses the host cell (transcription and translation) machinery to make new viral RNA and proteins for production of new viruses which bud off of the cell membrane (Benedict, 1994).

Reverse transcription is the process of converting the single strand RNA template into double stranded DNA using the enzyme reverse transcriptase (an RNA dependent DNA polymerase) (Figure 4). tRNA binds to the PBS site of the template RNA and acts as a primer for reverse transcriptase to initiate DNA synthesis. After the first strand of DNA has been synthesized, another enzyme associated with reverse transcriptase, RNase H, degrades the RNA template except for the PPT sequence. The PPT sequence acts as a primer for synthesis of the second strand of DNA from the first strand of DNA. Thus, the retrovirus genome is completely converted to double stranded DNA with the sequence: U3-R-U5-pbs-ψ-gag-pol-env-ppt-U3-R-U5. U3-R-U5 is the long terminal repeat (LTR) which contains the promotor and enhancer sequences, the initiation site for RNA synthesis, and the poly(A) tail signal (Brown *et al.*, 1987)

Integration of the proviral DNA into the host cell chromosome is done by the viral integrase, usually in regions of target DNA that are transcriptionally active (Rohdewohld *et al.*, 1987) Once integration is complete, the provirus uses the cellular transcription and translation enzymes to generate new viral progeny. mRNA for gag, pol, and env are produced and transported to the cytoplasm to make viral structural proteins and enzymes. Gag and pol polyproteins are produced from an unspliced mRNA that
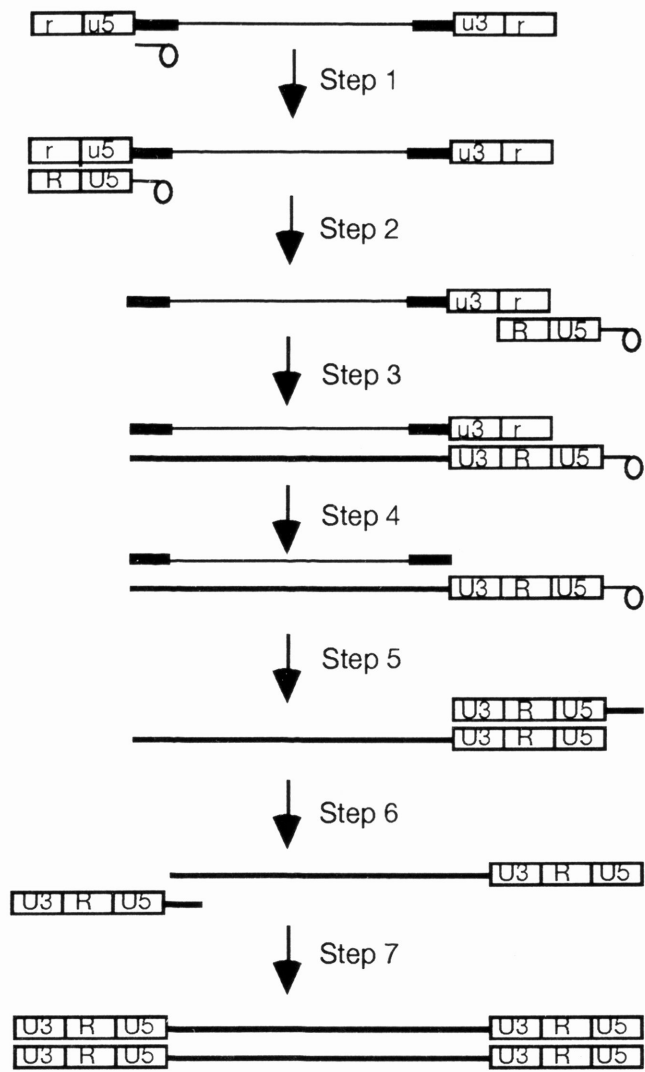
Figure 4. Reverse transcriptase model. Step 1. tRNA binds to PBS on RNA genome. Step 2. reverse transcriptase (RT) extends tRNA primer, removal of r-u5 region of RNA template occurs, and the first rearrangment of RNA strands takes place. Step 3. extension of the first strand of DNA by RT. Step 4. RNase H degrades u3-r of RNA template. Step 5. RT synthesizes U3-R-U5 region on 3' end of RNA template, and RNase H degrades rest of RNA template. Step 6. duplex melted and reformed. Step 7. RT synthesizes and completes second strand of DNA.

is identical to the viral RNA genome, and an envelope polyprotein is generated from a single spliced mRNA devoid of the gag and pol sequences (Coffin, 1990).

Assembly of a retrovirus is a complex but coordinated process that requires the gathering of capsid precursor proteins, virus genomic RNA, replicative enzymes, cellular tRNA, and glycoproteins to a common assembly site on the plasma membrane of the host cell. The gag gene plays a crucial role in this assembly. The assembly process of new retroviruses involves three steps: 1). gag polyproteins recognize the packaging signal (Ψ) on the viral RNA genome and package the RNA alone with the necessary replicative enzymes and tRNA primers into a pro-virion; 2). the pro-virion acquires its envelope through budding off of the host cell membrane; and 3). the pro-virion becomes an infectious mature virus after release. Release of the virion does not involve cell death (non-lytic) as seen with other viruses (i.e. vaccinia virus infection) (Li, 1995).

Retroviruses have been explored as vehicles for transferring foreign genes into mammalian cells for the purpose of human gene therapy due to their unique features (Gilboa, 1986). These unique features are: 1) a retrovirus can stably integrate its DNA genome into a cell chromosome and pass the genetic information encoded within from mother cell to daughter cell during replication; 2) a retrovirus replicates through a DNA form which is easier to manipulate than RNA; 3) the structural genes of a retrovirus, provided in trans, can be deleted from the virus without affecting replication - therefore the resulting virions are infectious but

replication defective; 4) most retroviruses infect cells without killing them or imparting any damage to functions of the infected cell; 5) retroviruses easily infect the host cell and therefore are more effective (100%) for gene delivery; and 6) retroviruses are structurally simpler than other vector capable viruses. When engineered as a retroviral vector for gene transfer, they do not produce viral proteins which may cause cell death or malfunctions (Li, 1995).

Retroviral vectors

A schematic of a retroviral vector system is shown in Figure 5. First, foreign genes are cloned into a retroviral vector which is derived from a retrovirus by deleting its structural genes. As a result, the recombinant retrovirus is replicative defective. Secondly, the recombinant retrovirus is introduced into cells which supply the retroviral structural proteins so that infectious recombinant retroviruses can be propagated. The cells that support the production of recombinant retroviruses are called packaging cells. The recombinant viruses are harvested from the packaging cells and used to infect target cells (Li, 1995).

The retroviral vector system contains two parts. The retroviral vector and the packaging cell line. The retroviral vector most used and frequently studied is moloney murine leukemia virus (MoMuLV). For the purpose of gene transfer, the retroviral vector has a minimum of two long terminal repeats containing sequences for proviral integration, a primer binding site and a packaging signal. Also, the retroviral vector has a selection
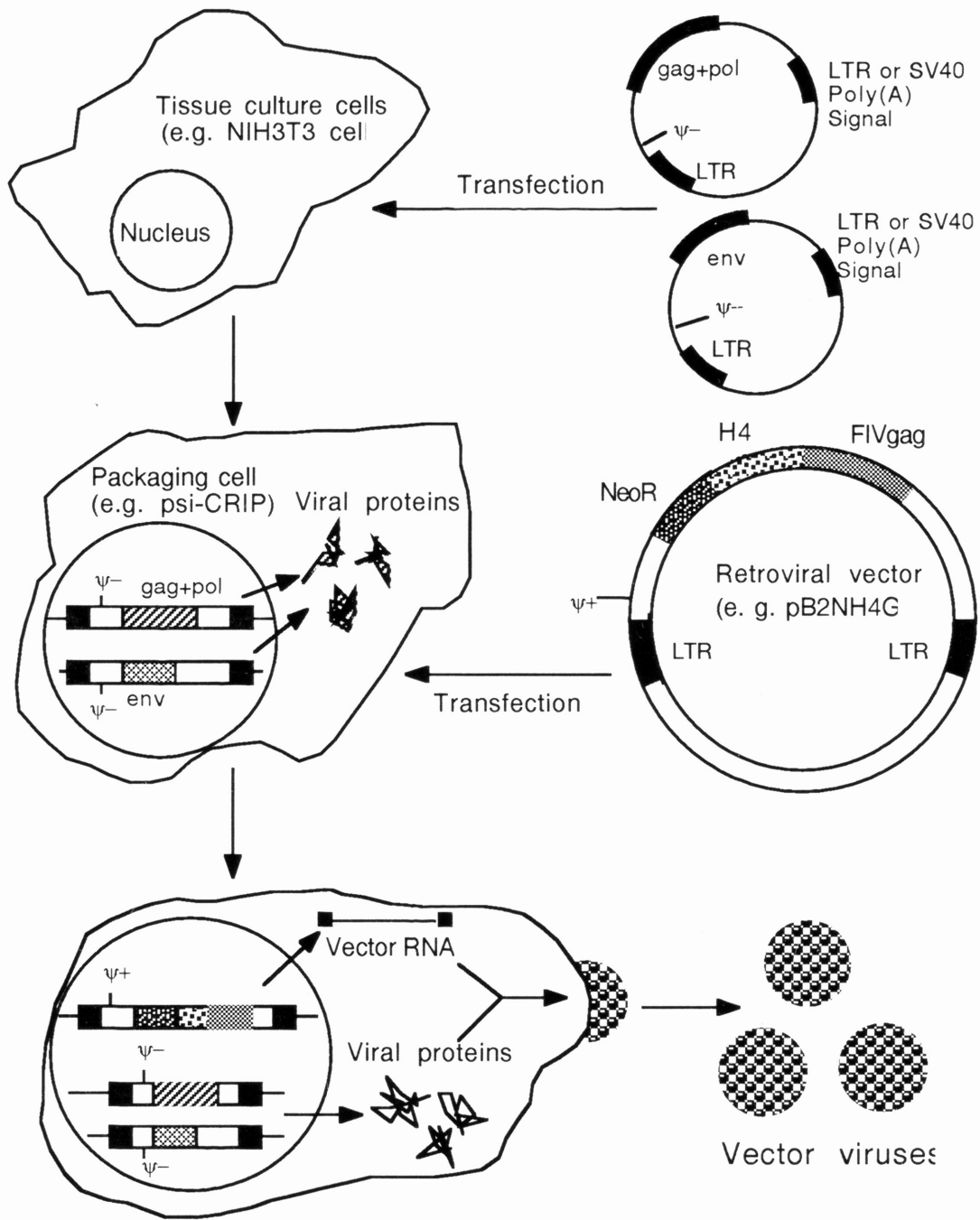
Figure 5.   Schematic of a retroviral vector system.

marker to positively select cells that are transfected with the vector. One of the most commonly used selection markers is the neomycin resistance (NeoR) gene which encodes neomycin phosphotransferase, an enzyme that modifies and detoxifies the antibiotic G418 which is toxic to many mammalian cells. The retroviral vector must also have cloning sites for insertion of genes of interest. The second part of the retroviral vector system, the packaging cells, provides the necessary replicative devices and structural proteins in trans for virion production and assembly, varying strategies for production of high titers of vector viruses, and infection of a wide range of species without allowing the possibility of replication competent viruses (Li, 1995).

In 1984, when gene therapy became a big issue in correcting genetic diseases, retroviral vectors came to be considered for the task of carrying modified genes to human somatic cells (Anderson). Today, more than thirty genetic disorders are being considered for gene therapy, some of which are adenosine deaminase deficiency (ADA), globulin deficiency and low-density lipoprotein (LDL) receptor deficiency (van Beusechem and Valerio, 1992). Other diseases such as cancer and HIV are also being considered for treatment using retroviral vector based gene therapy (Gilboa, 1990). With the widespread onset of AIDS and the lack of treatment in the form of drugs and vaccines, the use of retroviral vectors to treat HIV through gene therapy offers an attractive alternative. Several stages in the HIV life cycle can be targeted, such as blocking HIV from entering a cell by expressing the viral

glycoproteins in the cell with a retroviral vector (Li, 1995). But another alternative offered by the use of retroviral vectors has presented itself - using retroviral vectors to develop subunit vaccines. One study done with mouse fibroblasts transduced with a retroviral vector expressing HIV envelope glycoprotein - injected into BALB/c mice - showed induced HIV-specific cytotoxic T lymphocytes (CTL), as well as antibody responses (Warner et al., 1991).

Feline immunodeficiency virus (FIV)

Feline immunodeficiency virus (FIV) is a member of the subfamily lentivirinae of Retroviridae. It was first discovered in 1987 in a cat diagnosed with severe immunodeficiency in Petaluma, California (Pedersen et al., 1987). Since then, more than 34 genetically distinct strains of FIV have been isolated (Maki et al., 1992; Rigby et al., 1993; Sodora et al., 1994)

In comparison to other lentiviruses, FIV (Figure 6) resembles them in morphology, magnesium dependent reverse transcriptase activity, T cell tropism, and protein structure (Pedersen et al., 1990). Although FIV is more closely related to animal lentiviruses than primate lentiviruses, clinical symptoms and immunopathology of FIV infection very closely resemble HIV infection (Pedersen, 1990). Like HIV, FIV also infects CD4$^+$ and CD8$^+$ lymphocytes in vitro (Brown et al., 1991) and in vivo (English et al., 1993), although in vivo, the primary target of FIV infection is feline T lymphocytes (Pedersen et al., 1987). Recently, the receptor for FIV has been identified to be the homologue of feline CD9 (Willett
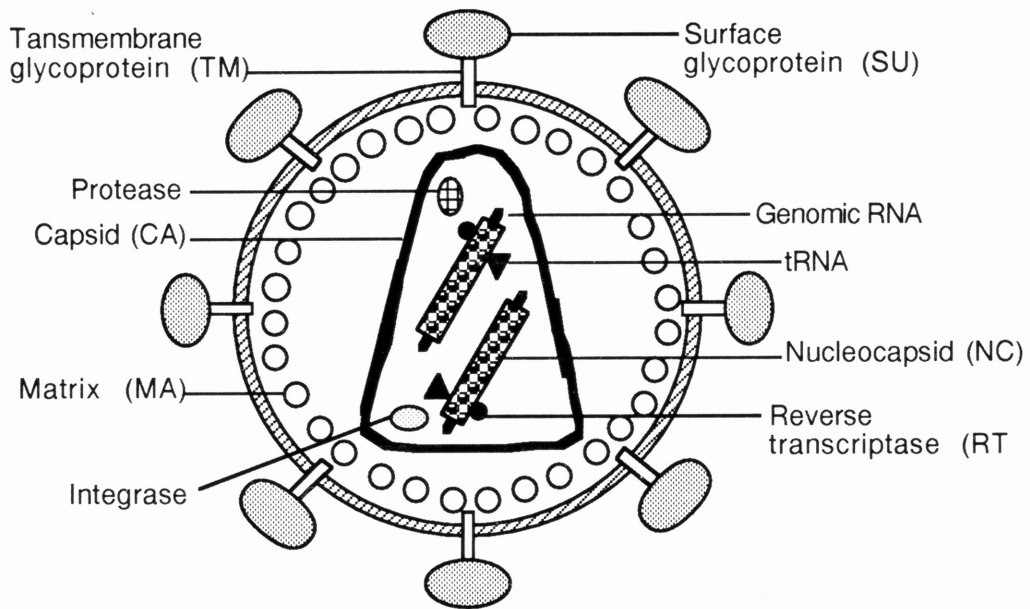
Figure 6.   Model of FIV.

et al., 1994). FIV has not been found to replicate in primary human, mouse, or dog cells (Yamamoto, *et al.*, 1988)

FIV's genetic structure (Figure 7) is typical of lentiviruses, with a genome of approximately 9.4 kb (Talbott *et al.*, 1989). The genome, like other retroviruses, contains three major structural genes: gag, pol, and env. The gag gene codes for a precursor core polyprotein (p50) which is cleaved after translation to form the matrix protein (p15), the capsid protein (p24), and the nucleocapsid protein (p10). The pol gene is translated with a ribosomal frameshifting mechanism, which produces a gag-pol fusion protein which is then cleaved to release a protease (p14), reverse transcriptase (p62), and integrase (p31). The env gene codes a precursor polyprotein which is cleaved into a transmembrane glycoprotein (TM) (gp36) and a surface glycoprotein (SU) (gp100) (Li, 1995).

The life cycle of FIV is like that of other retroviruses, though gene expression of the FIV provirus is not well defined, though it is thought to resemble that of HIV. Once the new virions are assembled and released, the pathogenesis of FIV infection in cats closely resembles HIV infection in humans. It occurs in three sequential stages: a transient primary illness occurring several weeks after infection, a sub-acute clinical stage, and a terminal AIDS stage. The primary stage is characterized by fever, neutropenia, lymphadenopathy, diarrhea, and increased susceptibility to secondary bacterial and viral infections (Yamamoto *et al.*, 1988; Barlough *et al.*, 1991). After the disappearance of the first phase, the cats can remain clinically
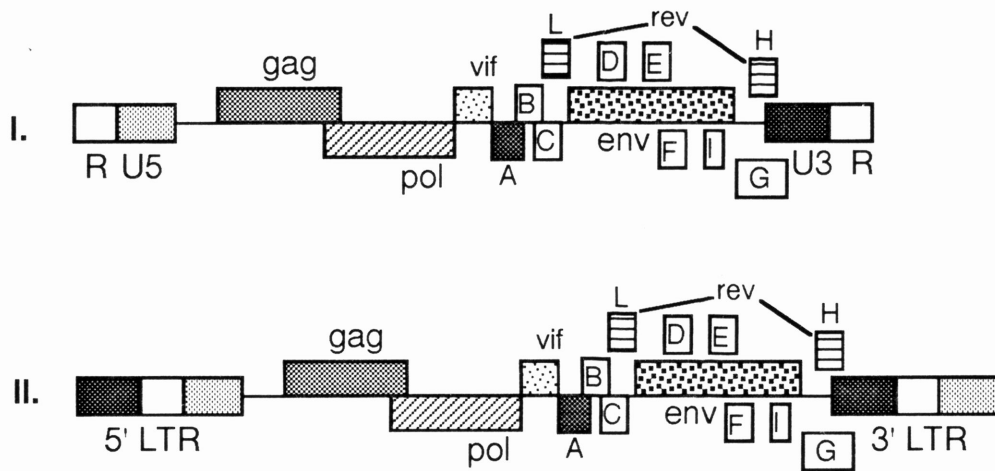
Figure 7. Genomic structure of FIV. I. FIV genome (RNA). II. FIV provirus genome. Small ORFs are labeled with A, B, C, D, E, F, G, H, and I.

normal for several years although they remain immuno-compromised as shown by decreased CD4$^+$/CD8$^+$ ratios (Barlough *et al.*, 1991). The terminal phase is characterized by wasting, diarrhea, lymphoid atrophy, secondary infections, neurological disease, lymphomas, and eventually death (Pedersen *et al.*, 1987). Transmission of the disease is mainly through bites and exchange of infected saliva. Free roaming cats are at more risk than indoor cats (Li, 1995).

Retroviral vectors as tools for studying CTL responses against individual FIV proteins

Since the onslaught of AIDS, scientists have searched for an effective vaccine to control the spread of HIV. Initially, efforts to develop a preventative vaccine against lentiviral infection have been based on the concept that a humoral antibody response would be required for protection. However, as discussed earlier, cell mediated immune response featuring the production of cytotoxic T lymphocytes may be the key in providing protection against lentiviral infections.

Cell mediated immunity is a critical component for host protection against viral infections, therefore a rational strategy for developing a protective vaccine should identify antigens which induce protective cell mediated immunity, especially cytotoxic T lymphocytes (CTL). Cell mediated immunity (involving CTL) that results in the elimination of infected cells is produced only in response to antigen that is endogenously expressed in target cells (Brodsky and Guagliardi, 1991).

Endogenous expression of an antigen can be achieved through

use of viral vectors, such as the retroviral vector (Miller *et al.*, 1989). The retroviral vector allows stable integration of its genome into the cellular chromosome and is not lytic. Therefore, the retroviral vector transduced cells can be maintained as stable cell lines for long term antigen expression. The retroviral vector also does not express proteins of the original retroviruses as seen with other retroviral vectors. And in studying CTL responses against individual antigens of a retrovirus, retroviral vector mediated gene transfer has the additional advantage of mimicking a retrovirus infection (Li, 1995).

With the commonalities seen between HIV and FIV, the use of FIV as an animal model for HIV is very important because it uses a small animal, poses no risk to humans, has biochemical properties and cell tropism that resemble those of HIV, and causes a clinically relevant disease in cats that is very similar to AIDS in humans. Therefore, studying FIV-specific CTL responses through construction of a retroviral vector producing transgenic cells expressing FIV viral proteins may provide useful information for HIV research. This thesis deals with the construction of a retroviral vector carrying the FIV *gag* protein p10 for production of transgenic cells that express this protein for studying FIV nucleocapsid-specific T-cell responses.

CONSTRUCTION OF A RETROVIRAL VECTOR

Introduction

To study the FIV *gag* nucleocapsid (p10) protein CTL response, the *gag*-protein, p10, of the Petaluma strain of FIV was chosen for insertion into the retroviral vector LXSN. The nucleocapsid protein is 266 bp (1713 to 1979) long and will be amplified using polymerase chain reaction (PCR) using synthetic oligonucleotide primers constructed on Oligo 4.0. The primers which are 28 nucleotides long, (the sequence to be amplified will be 364 bp long (1668 to 2031)) were constructed so that: the upstream primer contains an EcoRI site, as well as a start codon in frame (5' ATA**GAATTCC**CAGGATATAAA**ATG**CAAC 3'); the downstream primer contains a BamHI site (5' AGT**GGATCC**GGCCTTTTTTCTAATGTTG 3').

LXSN was developed by Miller and Rosman (1989) from the N2 vector with some modifications. The 5' LTR and sequences upstream of the MoMuLV were replaced with the homologous region from moloney murine sarcoma virus (MoMuSV); the start codon of MoMuLV gag on N2 was mutated to a stop codon; the MoMuLV env sequence and some non-coding regions of NeoR were removed to eliminate the possibility of generating competent helper virus by homologous recombination between the vector and the defective helper virus used in making the packaging cells; the NeoR gene was placed under the control of the SV40 promotor; and a multiple cloning site was included upstream of the SV40 promotor and NeoR gene so that foreign genes are expressed from the vector LTR (Li, 1995).

Materials and Methods

The plasmid, pUC119, containing the provirus of FIV Petaluma strain was provided by John H. Elder of the Scripps Research Institute, La Jolla, California. The plasmid, pLXSN, was provided by A. Dusty Miller of the Fred Hutchinson Cancer Research Center in Seattle, Washington. Aliquots of each were placed in separate 150 ml flasks of LB medium and cultured at $37^{\circ}C$ overnight. 15 mls of each culture were centrifuged at 4000 rpm for 10 minutes and the supernatant was discarded. Cells were resuspended in 1 ml of cell suspension (25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0)) and lysed by addition of 2 ml of cell lysis solution (0.2 N NaOH, 10% SDS), followed by addition of 1.5 ml of neutralization solution. The cell solution was then centrifuged at 8000 rpm for 15 minutes. The supernatant was removed to a fresh tube and 50 microliters of RNAse A added and incubated at $37^{\circ}C$ for 10 minutes. Phenol/chloroform extraction was preformed on the solution and centrifuged for 10 minutes at 7000 rpm. DNA was precipitated with 2.5 volume of ethanol and collected by centrifugation at 7000 rpm for 30 minutes. The supernatant was discarded and the DNA pellet was resuspended in 0.5 ml of 1X TE buffer and stored at $-20^{\circ}C$.

Amplification of the nucleocapsid protein from the pUC119 plasmid was accomplished by polymerase chain reaction (PCR). The 100 microliter PCR reaction contained the following reagents: 2.5 mM $MgCl_2$, 0.2 mM dNTP, 10X PCR buffer, .5 units of Ultima Taq DNA polymerase, 5 μl pUC119 template, and 1 μl of each FIV p10 specific primers. The primers, as discussed earlier were constructed using Oligo 4.0 and synthesized by the Advanced DNA Technologies

Laboratory at Texas A & M University in College Station, Texas. Twenty-five amplification cycles (95°C for 1 minute, 50°C for 30 seconds, and 72°C for 30 seconds) were performed on the Genetic Thermal Cycler (Model GTC-1, Precision Scientific). The resulting amplified product was analyzed by agarose gel electrophoresis and examined under UV light after staining the gel with ethidium bromide.

To prepare the retroviral vector, pLXSN, for insertion of the amplified p10 PCR product, they were each digested with the restriction enzymes EcoRI and BamHI in a 80 μl reaction to liberate complimentary sticky ends. Phenol/chloroform extraction was done to remove the restriction enzymes after 3 hours incubation at 37°C. DNA was precipitated by 2.5 volume of ethanol and stored at -20°C.

Ligation of the p10 enzyme digested fragment into the enzyme digested retroviral vector pLXSN was carried out in a 20 μl reaction of: 3 μl 10X ligation buffer, 2 units of T4 DNA ligase, 13 μl p10 fragment, and 2 μl pLXSN DNA. The mixture was then incubated at 12°C overnight. 10 μl of the ligation mixture was then used to transform INValphaF' Competent E. coli cells with the procedure provided by Invitrogen. The competent cells (50 μl aliquot) were thawed on ice and 2 μl of 0.5 M 2-mercaptoethanol and the ligation reaction aliquot was added. The cells were incubated on ice for 30 minutes, then heated for 45 seconds at 42°C and immediately placed back on ice for 2 minutes. 450 μl of pre-warmed SOC medium was added to the reaction and the entire solution was transferred to a 3 ml tube for incubation in a 37°C shaking

water bath for 1 hour. The cells were then plated on LB agar plates and grown overnight in a 37°C incubator.

Small scale DNA recovery (minipreps) were done to determine which colonies contained the pLXSN with the p10 fragment (pLGSN). Two milliliters of transformed E. coli, grown overnight in LB medium, were pelleted at 2500 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 200 μl of STET (10 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0), 5% Triton, 0.1 M NaCl) buffer before adding 20 μl of lysozyme (10 g/ml) solution. The bacterial suspension was incubated for 5 minutes at room temperature, then heated in a boiling water bath for 45 seconds. The bacterial lysate was then spun in a microcentrifuge at 12,000 rpm for 15 minutes. The gelatinous pellet was removed and the recombinant DNA was precipitated by adding 2 to 3 volume of ethanol. The plasmid DNA was collected by centrifuging at 12,000 rpm for 15 minutes and then resuspended in 100 μl of 1X TE buffer and stored at -20°C.

Identification of colonies containing the retroviral vector pLGSN was done by PCR using the same method as described above for the initial amplification of the p10 fragment from pUC119. Ten microliters of each PCR sample was analyzed by agarose (1.3%) gel electrophoresis and visualized under UV light after staining the gel with ethidium bromide.

Results

The construction of the retroviral vector pLGSN is shown in Figure 8. pUC119-FIV plasmid DNA and pLXSN vector DNA were first
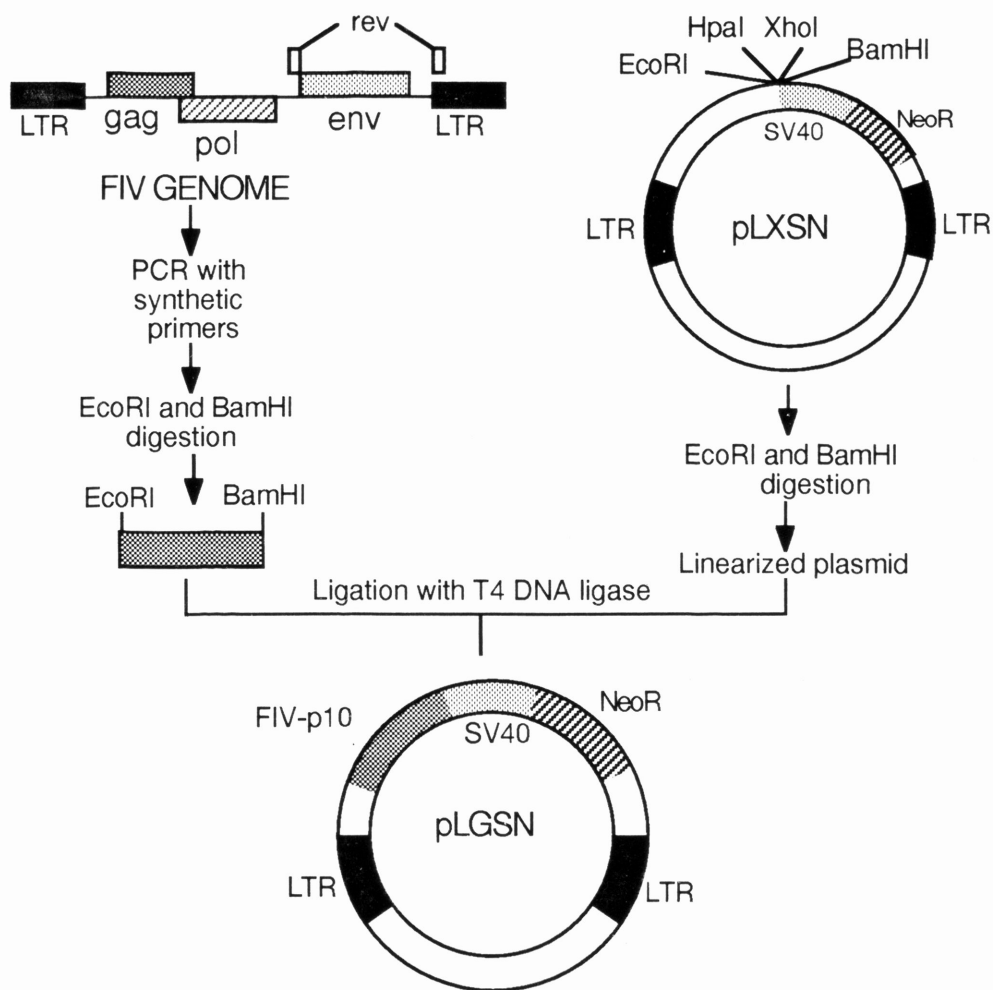
Figure 8. Construction of pLGSN with the FIV *gag* protein p10. The FIV p10 protein (nucleocapsid) sequence was amplified by PCR from the FIV genome in a pUC119 plasmid using synthetic primers: the upstream primer contained an EcoRI site; and the downstream primer contained a BamHI site. The ends were liberated by EcoRI and BamHI digestion and the p10 fragment was then inserted into the EcoRI-BamHI site of pLXSN producing pLGSN.

isolated from the LB cultures using large scale DNA recovery (maxipreps) procedures. Amplification of the p10 protein from the pUC119 plasmid was accomplished by PCR using the synthetic primers constructed for isolating the fragment. In order to insert the p10 fragment into the retroviral vector, the amplified p10 PCR product and pLXSN were digested with the restriction enzymes EcoRI and BamHI. Ligation and transformation was accomplished, producing forty transformed colonies. Possible pLGSN DNA was isolated using miniprep procedures and the colony samples were then examined by PCR for identification of which colonies were recombinants. Agarose gel electrophoresis of the PCR samples showed no recombinant retroviral vectors.

Discussion

After completing PCR with the pUC119 plasmid, agarose gel electrophoresis showed amplification of the 364 bp nucleocapsid fragment. Subsequent restriction digest with EcoRI and BamHI of the p10 PCR fragment and the retroviral vector pLXSN for ligation and transformation of competent cells provided forty transformed colonies. PCR analysis of these colony DNA did not show the expected amplification of the p10 fragment when examined on an agarose gel. Analysis of an aliquot of the purified restriction digested p10 fragment by agarose gel electrophoresis showed the fragment to be of approximately 200 bp instead of the expected 364 bp (Figure 9).

Re-evaluation of the nucleocapsid sequence revealed a natural EcoRI site at base pair 1871. The presence of this site meant that
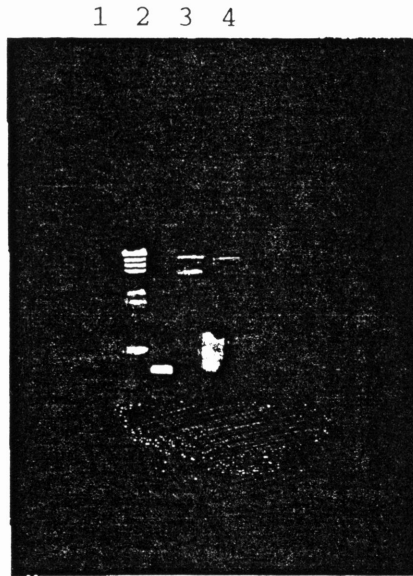
Figure 9. Agarose gel analysis of purified EcoRI and BamHI digested p10 protein. Lane 1. Lambda size marker (23kb, 9kb, 6kb, 4kb, 2.2 kb, 2 kb, and 500bp [from top to bottom]). Lane 2. purified restriction digested p10 fragment. Lane 3. purified restriction digested pLXSN. Lane 4. aliquot of ligation reaction of p10 into pLXSN. The p10 digested fragment runs at @ 200 bp instead of the expected 364 bp.

in the EcoRI and BamHI digestion of the amplified p10 PCR product to liberate the sticky ends for insertion into the retroviral vector, the p10 fragment was being cut into two pieces: an EcoRI flanked 204 bp sequence, and a 5' EcoRI and 3' BamHI flanked 160 bp sequence. While the 160 bp fragment would be inserted into the retroviral vector, subsequent analysis by PCR with the initially constructed primers would not yield amplification of the sequence as only the downstream primer would anneal to the template DNA.

This led to reconstructing the upstream primer with an XhoI site instead of an EcoRI site: 5' ATAGG**CTCGAG**AGGATATAAA**ATG**CAAC 3'. Research will be done as described above and outlined in Figure 8, but using this upstream primer along with the original downstream primer to amplify the p10 sequence from the pUC119 plasmid. Restriction digest to liberate the complimentary sticky ends of the p10 fragment and pLXSN will be done with XhoI and BamHI. PCR of transformed colony DNA or XhoI and BamHI digestion will be used to determine which colonies contain the desired retroviral vector pLGSN. These colonies will then be used in transfecting packaging cells for production of CTL responses to the FIV-*gag* protein p10.

Construction of a Retroviral Vector
with a Partial p10 Sequence

Introduction

Further studies on fragmented FIV-*gag* proteins is also desirable in understanding cytotoxic T lymphocyte responses to endogenously expressed foreign antigens for production of a protective vaccine against lentiviral infections. Therefore, the 204 bp fragment generated by the EcoRI digestion of the amplified p10 PCR product will be inserted into the retroviral vector pLXSN.

Materials and Methods

The p10 sequence amplified by PCR and digested by EcoRI and BamHI was retained for use in this set of experiments. The retroviral vector pLXSN was digested with the restriction enzyme EcoRI in a 80 μl reaction to liberate complimentary sticky ends. Phenol/chloroform extraction was done to remove the restriction enzymes after 3 hours incubation at 37°C. DNA was precipitated by 2.5 volume of ethanol and stored at -20°C.

Ligation of the 204 bp p10 fragment into the restriction digested retroviral vector was preformed as described earlier. Transformation was also performed as described above. The resultant 60 transformed colonies were minipreped as before to recover the DNA, and stored at -20°C.

To determine which colonies contain recombinant retroviral vector DNA, 25 μl aliquots of the miniprep samples were digested with 2 units of EcoRI in a 30 μl reaction at 37°C for 3 hours.

Results

The 204 bp p10 fragment generated by restriction digest with EcoRI and BamHI from the first discussion was inserted into the EcoRI digested retroviral vector. Transformation of E. coli competent cells produced a large quantity of transformed E. coli colonies. Sixty colonies were minipreped and 25 μl aliquots of forty of these samples were digested with EcoRI.

Discussion

Successful ligation and transformation of the 204 bp p10 fragment into the retroviral vector pLXSN generated a enormous quantity of transformed colonies. At the time of this paper, a polyacrylamide gel is being used to analyze the EcoRI digestion of forty of the transformed colonies to determine which colonies contain the recombinant retroviral vector.

Generation of the retroviral vector, pLGSN, with both the intact p10 sequence and the 204 bp p10 fragment are expected at high success rates. Other work being done in this lab by Yuan Xu with the FIV-*gag* matrix protein has yielded a 100% recombination of the matrix protein and the retroviral vector using the same procedures. Once generation of the retroviral vector pLGSN with the nucleocapsid (and with the nucleocapsid fragment) is determined, the transformed colonies containing the recombinant retroviral vector will be sequenced and used to transfect Ψ-CRE and Ψ-CRIP packaging cell lines for infection of feline T cell blasts for studying FIV nucleocapsid-specific T-cell responses.

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