STUDIES OF VIRAL TETHERING AND ITS EFFECT IN THE EARLY PHASE OF MLV LIFE CYCLE

by

ARABA ADDAQUAY

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ABSTRACT OF THE THESIS

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By ARABA ADDAQUAY

Thesis Director:

Monica Roth

The p12 proteins designed to encode peptide sequences from viral tethering proteins was shown to rescue murine leukemia virus (MLV) p12 PM14 lethal mutants (Schneider et al., 2013). However, the viral tethering protein from Kaposi's sarcoma associated herpes virus (KSHV) latency-associated nuclear antigen encoding a nuclear localization signal, $LANA_{(1-32)}$, could not rescue MLV p12 PM14. This isolate p12 with $LANA_{(1-32)}$ fused to GFP was found to bind tightly to mitotic chromosomes. We show that virus bearing p12 $LANA_{(1-32)}$ PM14 retain the group-specific antigen (Gag) precursor inside the nucleus and that insertion of a nuclear export signal (NES) is able to counterbalance the effect of the NLS (nuclear localization signal). However, viral kinetics of MLV p12 $LANA_{(1-32)}$ PM14 with or without the NES indicates that the virus is still not viable. This suggests that the original hypothesis of the tight tethering domain that is p12 $LANA_{(1-32)}$ is detrimental to MLV infection.

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Chapter 1: INTRODUCTION

The retroviral life cycle is defined by two events, the early and late phases. In the early phase, the virus binds with its Envelope protein to the receptor on the target cell, there is fusion of the viral and cellular lipid membranes and the release of the virus core into the cytoplasm of the host cell. In the virus core, the viral RNA genome is reverse transcribed into double stranded (ds) DNA within a reverse transcription complex (RTC) composed of reverse transcriptase (RT), integrase (IN), capsid (CA), p12, nucleocapsid (NC), and cellular proteins (Fassati et al., 1999; Maetzig et al., 2011). The RTC transitions into a pre-integration complex (PIC) and in murine leukemia virus (MLV), the trafficking of the PIC to host chromatin is facilitated by nuclear envelope breakdown during mitosis, unlike in human immunodeficiency virus (HIV), which goes through the nuclear pore (Roe et al., 1993; Elis et al., 2012). Integration of the ds viral DNA into host chromatin is mediated by the PIC and this marks the end of the early events of the retroviral life cycle. The late phase is marked by the replication of the provirus (integrated ds viral DNA). This may result in an unspliced Gag/Pol precursor protein or the spliced Gag precursor protein, which assemble at the plasma membrane along with envelope proteins and buds off to give the immature virion. Cleavage of the precursors by viral protease into their constituent monomers gives the infectious particle, which can infect other cells, starting the cycle once again (Figure 1).

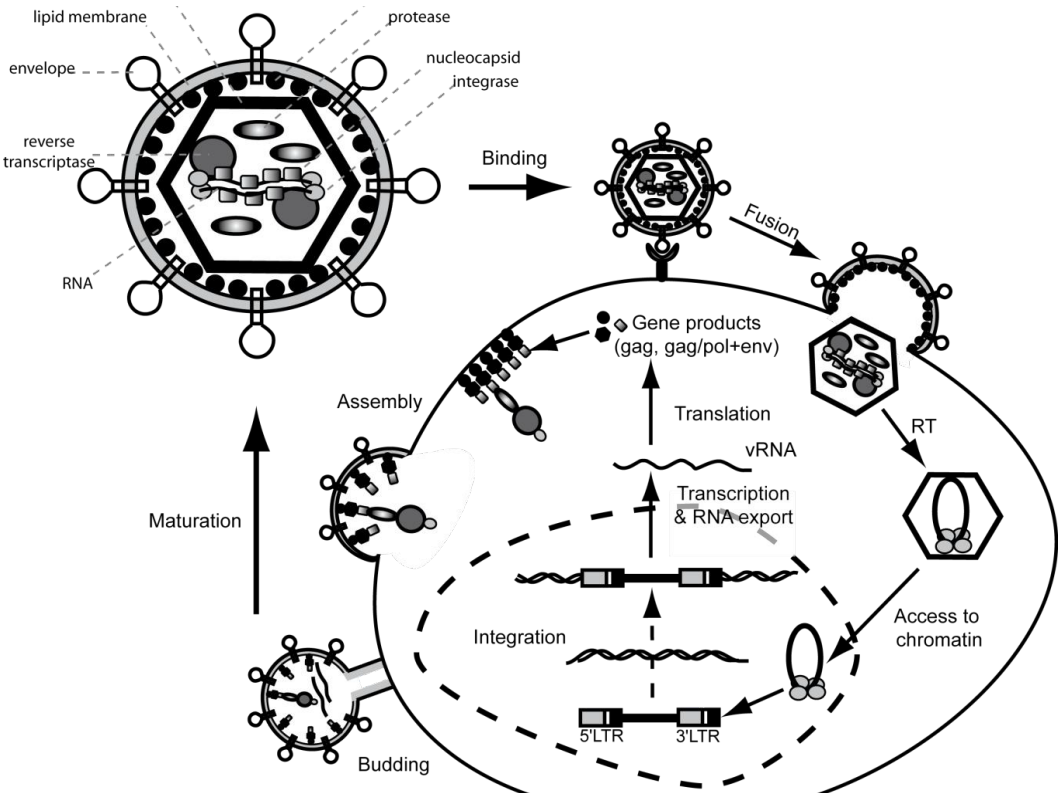


Figure 1: MLV life cycle beginning with viral entry, fusion, and uncoating to give the virus core, reverse transcription, trafficking of the pre-integration complex (PIC) to nucleus, integration, viral RNA replication, assembly, budding, and maturation.

Citation: Tobias Maetzig, Melanie Galla, Christopher Baum and Axel Schambach. Gammaretroviral Vectors: Biology, Technology and Application. *Viruses* 2011, *3*, 677-713.

The Gag precursor is composed of matrix (MA), p12, capsid (CA), and nucleocapsid (NC) (Fassati et al., 1999). Over the years, there has been generated interest in the p12 protein and its function in the MLV life cycle. As a component of the PIC, p12 has been shown to function in early and late events in the life cycle of MLV. The p12 protein has 84 amino acids and is very rich in proline, containing two polyproline motifs, the PPPY and PPPS. The late domain of p12 is attributed to the presence of the PPPY motif, and this motif functions in the assembly process, which is in the late phase of the viral life cycle. p12 was found to function in the early phase of the viral life cycle when deletion of p12 and mutations in the PPPY motif decreased virion

production (Yuan et al., 1999). In addition, alanine-scanning substitution mutations in the N and C-terminus of p12 (mutants PM5, PM7, PM13, PM14, and PM15) (Figure 2) demonstrated its role in retaining the virus in the nucleus (Yuan et al., 1999), and hence, its role in early stages of the retroviral lifecycle.

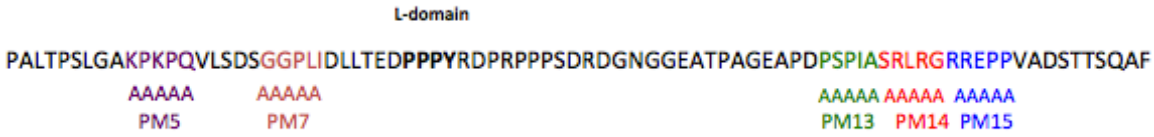


Figure 2: Amino acid sequence of p12. In colored scheme are the mutants made from alanine-scanning substitution mutations in the N-terminus (PM5,7) and C-terminus of p12 (PM13-15). Blocks of 5 amino acids were substituted with alanines and their viral kinetics tested (Yuan et al., 1999). Late domain is indicated in bold.

Later studies supported the p12 protein's involvement in binding viral DNA to host chromatin (Prizan-Ravid et al., 2010). Further experiments by Wight et al., 2012, found that the alanine-scanning substitution mutations in the PM13-15 (Yuan et al., 1999) regions affected chromatin binding. The PM14 mutation, which displays a negative RT-activity profile even after 24 days of transfection (Yuan et al., 1999), has been of particular interest to our study. p12-PM14 mutants defective in early stages of viral infection were rescued when alternative viral tethering domains from prototype foamy virus (PFV) and Kaposi's sarcoma associated herpes virus (KSHV) were inserted into the p12 region of MLV (Schneider et al., 2013). However, insertion of KSHV LANA$_{(1-32)}$ (latency-associated nuclear antigen encoding a nuclear localization signal) did not rescue MLV p12-PM14. The findings from this study showed an absence of reverse transcriptase activity in supernatants collected over a 40-day period from D17pJET cells transfected with MLV p12-KSHV LANA$_{(1-32)}$ PM14. In addition, they found p12- KSHV

LANA(1-32) PM14 to have strong tethering to mitotic chromosomes (Schneider et al., 2013).

Recent studies have shown the utility of nuclear export signals (NES) for export of proteins out of the nucleus and into the cytoplasm of the cell. Insertion of NES sequences within p12 and NC genes have been shown to localize Gag proteins to the cytoplasm (Wu et al., 2014). Significant conclusions from the data included the export of MLV Gag precursor protein out of the nucleus showing that NLS sequences were offset by the NES so that MLV fitness had very little overall change (Wu et al., 2014). We inserted the NES encoding NINELALKFAGLDL (Wu et al., 2014) between the C-terminus of matrix (MA) and the N-terminus of p12 into MLV p12- LANA(1-32) PM14 (Schneider et al., 2013) to counterbalance the NLS. The results of this study shows no reverse transcriptase activity in supernatants collected over a 40-day period from canine D17pJET cells transfected with MLV p12 LANA(1-32) PM14 with the NES. However, we were able to export Gag precursor out of the nucleus to produce infectious virions and also detected reverse transcription products comparable to WT virus in cells infected with virus produced from transient transfections. This explains that the defect in the viral replication cycle does not affect steps leading up to and including reverse transcription, but suggests that the tight tethering domain that is KSHV LANA(1-32) compromises successful infection.

Chapter 2: MATERIALS AND METHODS

2.1 Plasmid construction

All the vectors were constructed using pNCA-C (Felkner and Roth, 1999) proviral vector as backbone and mutations made in the p12-PM14 region are described below.

NCAC P12 LANA (1-32) PM14

This plasmid was previously named as KSHV p12-LANA (1-32) and was constructed identically in Schneider et al., 2013.

NCAC P12 LANA (1-32) PMI4 IN- NeoR

KSHV LANA (1-32) (Schneider et al., 2013) was inserted in pNCA-C IN- NeoR (IN mutant pNCA-C neomycin resistance plasmids) using restriction enzymes EcoRI and XhoI (Schneider et al., 2012).

NCAC NES P12 LANA (1-32) PM14 IN- NeoR

The NES sequence encoding NINELALKFAGLDL was inserted between the C-terminus of matrix (MA) and the N-terminus of p12 by first using primers EcoRI fwd (5'-CAAGAATTCTCATGTTTGACAGCTTAT-3') and NES p12 ov rev (5'-AAGATCAAGACCAGCGAACTTCAATG-3') on template pNCAC p12 LANA (1-32) PM14 (Schneider et al., 2013) to get the first PCR product (product 1), and then with primers NES p12 ov fwd (5'-AACATCAATGAACTGGCATTGAAGTT-3') and XhoI rev (5'-GCGCTCGAGGGGAAAAGC-3') to get the second PCR product (product 2). The PCR

products (product 1 and 2) were joined by overlapping PCR using primers EcoRI fwd and XhoI rev to give a third product (product 3). PCR product 3 and plasmid NCAC P12 LANA (1-32) PM14 IN- NeoR (described previously) were digested with EcoRI and XhoI restriction enzymes and ligated to give pNCAC NES P12 LANA (1-32) PM14 IN- NeoR.

NCAC NES PR site P12 LANA-NLS PM14 IN- NeoR

The protease site encoding RSSLY/PALTP followed by the NES sequence encoding NINELALKFAGLDL was inserted between matrix (MA) and p12 using primers EcoRI fwd (5'-CAAGAATTCTCATGTTTGACAGCTTAT-3') and NES-PRsite-p12 ov rev (5'-AAGATCAAGACCAGCGAACTTCAATG-3') on template pNCAC p12 LANA (1-32) PM14 (Schneider et al., 2013) to get the first PCR product (product 1), and then with primers NES-PRsite-p12 ov fwd (5'-ATCAATGAACTGGCATTGAAGTTCGCT-3') and XhoI rev (5'-GCGCTCGAGGGGAAAAGC-3') to get PCR product (product 2). Product 1 and 2 were then joining by overlapping PCR using EcoRI fwd and XhoI rev to get PCR product 3. Product 3 and plasmid NCAC P12 LANA (1-32) PM14 IN- NeoR (described above) were digested with restriction enzymes EcoRI and XhoI ligated to give pNCAC NES PRsite P12 LANA (1-32) PM14 IN- NeoR

NCAC NES P12 LANA (1-32) PM14

As described for NCAC NES P12 LANA (1-32) PM14 IN- NeoR, using template pNCAC p12 LANA (1-32) PM14 (Schneider et al., 2013).

NCAC NES PRsite P12 LANA (1-32) PM14

As described for NCAC NES PRsite P12 LANA (1-32) PM14 IN- NeoR, using template pNCAC p12 LANA (1-32) PM14 (Schneider et al., 2013).

NCAC NES P12

The NES sequence encoding NINELALKFAGLDL was inserted between the N-terminus of p12 and the C-terminus of matrix (MA) using primers EcoRI fwd (5'-CAAGAATTCTCATGTTTGACAGCTTAT-3') and NES p12 ov rev (5'-AAGATCAAGACCAGCGAACTTCAATG-3') on template vector pNCA-C (Felkner and Roth, 1999) to get the first PCR product, product 1. To get the second PCR product (product 2), we used primers NES p12 ov fwd (5'-AACATCAATGAACTGGCATTGAAGTT-3') and XhoI rev (5'-GCGCTCGAGGGGAAAAGC-3'). Product 1 and 2 were then joining by overlapping PCR using EcoRI fwd and XhoI rev to get PCR product 3. Products 3 and pNCAC were digested with restriction enzymes EcoRI and XhoI and ligated to give vector of interest, pNCAC NES P12.

NCAC NES PRsite P12

The NES sequence encoding NINELALKFAGLDL was inserted in 2 regions of the template vector pT-cmyc (Wu et al., 2014): first using primers EcoRI fwd (5'-CAAGAATTCTCATGTTTGACAGCTTAT-3') and NES p12 ov rev (5'-AAGATCAAGACCAGCGAACTTCAATG-3') first PCR product (product 1), and then

with primers NES p12 ov fwd (5'-AACATCAATGAACTGGCATTGAAGTT-3') and XhoI rev (5'-GCGCTCGAGGGGAAAAGC-3') to get the second PCR product (product 2). Products 1 and 2 were joined by overlapping PCR using EcoRI fwd and XhoI rev to get PCR product 3. Products 3 and pNCAC were digested with restriction enzymes EcoRI and XhoI and ligated to give vector of interest, pNCAC NES PRsite P12.

NCAC NES P12 IN- NeoR

As described above for plasmid NCAC NES P12, except ligating overlapping PCR product with vector backbone pNCA-C IN- NeoR (Schneider et al., 2012).

NCAC NES PRsite P12 IN- NeoR

As described above for plasmid NCAC NES PRsite P12, except ligating overlapping PCR product with vector backbone pNCA-C IN- NeoR (Schneider et al., 2012).

2.2 D17/EGIP Cell Line Generation

To generate stable D17 canine viral producer cell lines, 293T CeB cells plated on 60 mm dish the night before were transfected with 3.5 μg WT NCA-C NeoR (Schneider et al., 2012) or IN mutant pNCA-C-NeoR viral constructs with NES sequences, in addition to 3.5 μg pHIT-G plasmid DNA (VSV-G) using Lipofectamine reagent (Promega) for 5-6 hrs and then covered with fresh medium overnight (DMEM (Gibco) with 10% FBS (Atlanta Biologicals) and 1% antimycotic/antibiotic (Gibco)). The following day, the cells were treated with 10 mM Na-butyrate for 6 hours for Gag-Pol expression from the plasmid DNA, after which the cells were covered overnight with 5 mL fresh medium and

viral supernatants were collected the following day. D17/EGIP cells with puromycin resistance (Schneider et al., 2012) were plated on 60 mm dish and infected with the viral supernatants in combination with 8 μg/mL polybrene overnight, after which the medium was replaced. The cells were then selected for puromycin and neomycin resistance.

2.3 Confocal Microscopy

The D17/EGIP cell lines (described above) were set to adhere to poly-l-lysine coated glass slides, fixed and permeabilized with -20 °C methanol, after which they were blocked in 5% BSA solution overnight. For consecutive antibody studies, donkey antigoat IgG (Alexa Flour) primary antibody was used followed by goat anti-mouse (81S263) secondary antibody for an hour each. Following this, 20 μl Pro-Long Gold antifade reagent with DAPI was used to stain the cells. The images were examined on a Zeiss LSM510 META confocal microscope with a 63 × water immersion objective at the Robert Wood Johnson Medical School Confocal and Electronic Imaging Center.

2.4 DEAE Dextran transient expression and RT-assay

D17pJET cells, a canine osteosarcoma cell line expressing the ecotropic MLV receptor, mCAT, were transiently transfected with proviral DNA to determine the time course for viral replication. The day before transfection, $1x10^5$ D17pJET cells were split onto 60 mm dishes and cultured with fresh medium. The transfection was done in triplicate; 650 μl PBS+ was combined with 250 ng miniprep DNA and vortexed. 150 μl diethylaminoethyl (DEAE)-Dextran (1 mg/mL) was added and vortexed again. The cells were then washed twice with 2 mL PBS+ and aspirated, after which the DEAE-Dextran

and miniprep DNA mixture was added dropwise to the cells and the plate rocked to spread it. The cells were incubated at 37 °C for 30 minutes, rocking the plate every 10 mins. After 30 minutes, the cells were washed twice with 2 mL PBS+ and incubated overnight with 5 mL fresh medium. 3 days afterwards, when the plate was subconfluent, 100 µL of viral supernatant was collected and stored at -20 °C. The plates were split twice a week when confluent until reaching day 40, each time collecting 100 µL of viral supernatant to be tested for reverse transcriptase (RT) activity (Goff et al., 1981). Plates testing positive for RT-activity were maintained in cell culture; gDNA was extracted from these cells and sequenced for any reversions.

2.5 Lac-Z Viral Titer Assay and Western Blotting

293T cells plated on 60 mm dish to be 60% confluent the following day was transiently transfected with 1 µg proviral DNA, 1 µg VSV-G (p-HIT-G), and 1 µg RT43.2TnIsbgal plasmid (an MLV vector based genome containing the β-galactosidase gene and a packaging signal which we call here onwards placZ) (Ting et al., 1998) using FuGENE 6 reagent. The 293T cells were covered with 3 mL fresh medium overnight. The following day, the media was replaced and the cells treated with Na-butyrate for 6 hours, after which it was replaced with 3 mL fresh medium overnight for viral supernatant collection the following day. Canine D17 cells were split onto 35 mm dish (10^5 cells). The viral supernatant was filtered with 0.45 µm acrodisc (Pall Corportation) and stored at 4 °C. A modified ELISA assay (Wu et al., 2013) was performed on the filtered viral supernatant to equalize virus for infection. During the infection, 8 µg/mL polybrene was added. The following day, the media was replaced. The next day, the cells

were fixed with paraformaldehyde for 30 minutes, aspirated, washed with 2 mL PBS for 5 minutes, and then 1 mL PBS and then stained with X-Gal salt (Invitrogen) solution (1mg X-Gal / 25 μL DMSO).

To perform Western blotting, 1 mL each of the filtered viral supernatant was pelleted at maximum speed for 40 minutes at 4 °C. The pellet was suspended in 2x loading dye (LDS and β-mercaptoethanol), heated at 56 °C for 5 minutes, and loaded onto a 12% SDS-PAG, which was run at 150 V for about 40 minutes. The protein was transferred onto a Turbo Mini-gel transfer pack (Bio-Rad) for 7 minutes and first probed for p30 using primary antibody 81S263, followed by bovine anti-goat-HRP-conjugated secondary antibody and imaged using ECL Western Blotting Substrate (Thermo Scientific). Overnight, p12 was probed using anti-p12 antibody (ViroMed; 74S000430), followed by bovine anti-goat-HRP-conjugated secondary antibody.

2.6 qPCR

Infection onto human Te671 cells were normalized by qPCR by first transiently transfecting 293T cells with 2 μg proviral DNA and 2 μg p-HIT-G plasmid DNA (VSV-G) using FuGENE 6 reagent. The evening before transfection, 5 x 10^5 293T cells were plated onto a 10 cm dish. The day after transfection, the cells were treated with Na-butyrate for 6 hours, after which the medium was replaced for overnight viral collection. The viral supernatant was filtered with a 0.45 μM acrodisc (Pall Corportation) and stored at -80 °C. Viral RNA (vRNA) was extracted from the filtered supernatants using the Qiagen vRNA extraction kit. Complementary DNA (cDNA) synthesis of the vRNA was

made using random hexamers in accordance with Superscript III Reverse transcriptase (Invitrogen). Viral particles were assayed for minus strand strong stop intermediates using primers (5'-GCCAGTCCTCCGATTGACTG -3') and (5'-TGACGGGTAGTCAATCACTCAGAG -3'). The day before infection, Te671 cells were seeded onto 60 mm dishes (2 $x10^5$ cells). The following day, the cells were infected with viral supernatant (6.7 x 10^9 copies/mL) in a total volume of 4 mL per plate, with 8 μg/mL polybrene. 24 hours after infection, the infected cells were trypsinized and gDNA extracted in accordance with Qiagen DNeasy Blood & Tissue Kit and used for qPCR.

250 ng gDNA was used in the qPCR reaction using Power Sybr Green PCR Master Mix (Life Technologies). Each reaction was performed in triplicate. Minus strand strong stop intermediates using primers (5'-GCCAGTCCTCCGATTGACTG -3') and (5'-TGACGGGTAGTCAATCACTCAGAG -3') and plus strand extension intermediates using primers (5'-GCCAGTCCTCCGATTGACTG -3') and (5'-AATCGGACAGACACAGATAAGTTGC -3') were quantified (Schneider et al., 2012). MSSS and PSE products were normalized to RPPH1 gene using primers (5'-CGTGAGTCTGTTCCAAGCTC-3') and (5'-GGGAGGTGAGTTCCCAGAG-3).

Chapter 3: RESULTS

3.1 Design of MLV p12-PM14 mutant with NES insert

The amino acid sequence of MLV Gag p12 and the position where the NES sequence and the extra protease (PR) cleavage site were inserted into p12 PM14 are diagrammed in Figure 3. Previous experiments have demonstrated the tethering function of p12 using alternative tethering domains from PFV and KSHV LANA protein, which rescued p12-PM14 mutants. However, NCAC P12 LANA(1-32) PM14 could not rescue the p12-PM14 mutation, unlike NCAC P12 LANA(1-23) PM14. The difference between LANA(1-23) and LANA(1-32) being RKRNRSPER, was previously characterized as an NLS (Barbera et al., 2006). This lack of rescue of LANA(1-32) in Schneider et al., 2013, was attributed to the tighter tethering of the viral DNA to mitotic chromosomes in comparison to LANA(1-32). However, our initial results demonstrated localization of CA in the nucleus of D17/EGIP cells transfected with the vector NCAC P12 LANA(1-32) PM14 (Figure 6C) and we deduced two possible explanations based on these results: either the NLS sequence in p12 LANA(1-32) PM14 interfered with export of the Gag precursor out of the nucleus (late stage of infection) or that the tight tethering of p12 LANA (1-32) PM14 to mitotic chromosomes interfered with integration. For this reason, we inserted an NES encoding NINELALKFAGLDL (Wu et al., 2014) between the N-terminus of p12 and the C-terminus of matrix (MA) to counterbalance the NLS to export NCAC P12 LANA (1-32) PM14 Gag precursor out of the nucleus and into the cytoplasm to allow viral budding. Insertion of the NES at the beginning of p12 ensures the fusion protein NES p12 LANA (1-32) PM14 after Gag processing by protease (Figure 4), whereas inserting a protease cleavage site after the NES enables cleavage of the NES after

protease cleavage to release the p12 LANA (1-32) PM14 protein (Figure 5). To determine whether insertion of the NES affected the function of p12, controls NCAC NES P12 and NCAC NES PR P12 were constructed in the absence of LANA (1-32) (Materials and Methods), so that after Gag processing, we have Gag NES p12 and WT Gag p12 respectively.

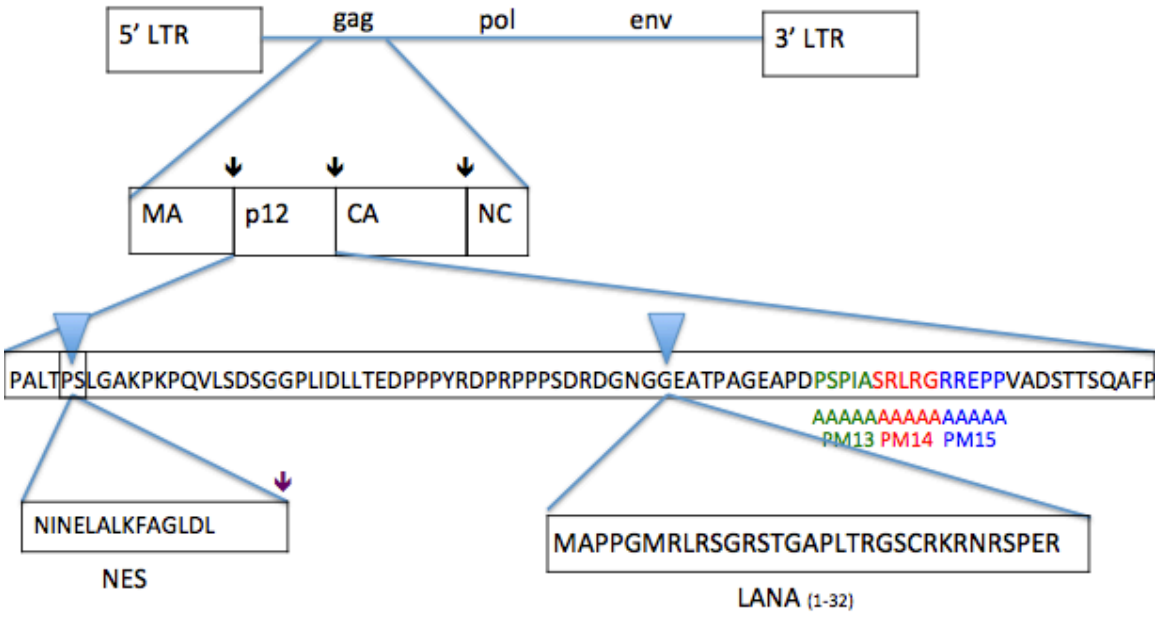


Figure 3: Schematic diagram of MLV open reading frame. Gag constituents include MA, p12, CA, and NC. Purple arrow indicates the new protease cleavage site added. The NES was inserted at the N terminus (PS), whereas LANA (1-32) was inserted at 49G (LANA(1-32) replaces 49G). An extra protease cleavage site was included for PR NES LANA (1-32).

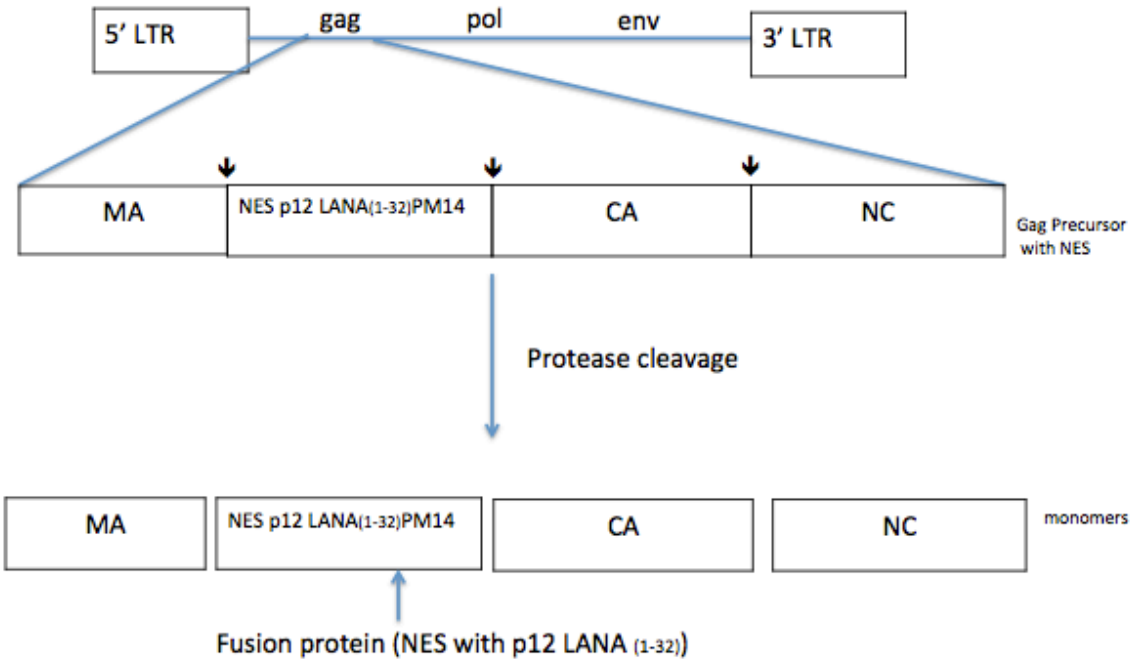


Figure 4: Schematic representation of Gag processing after protease cleavage to give fusion protein NES p12 LANA(1-32) PM14. Black arrows represent protease cleavage sites.

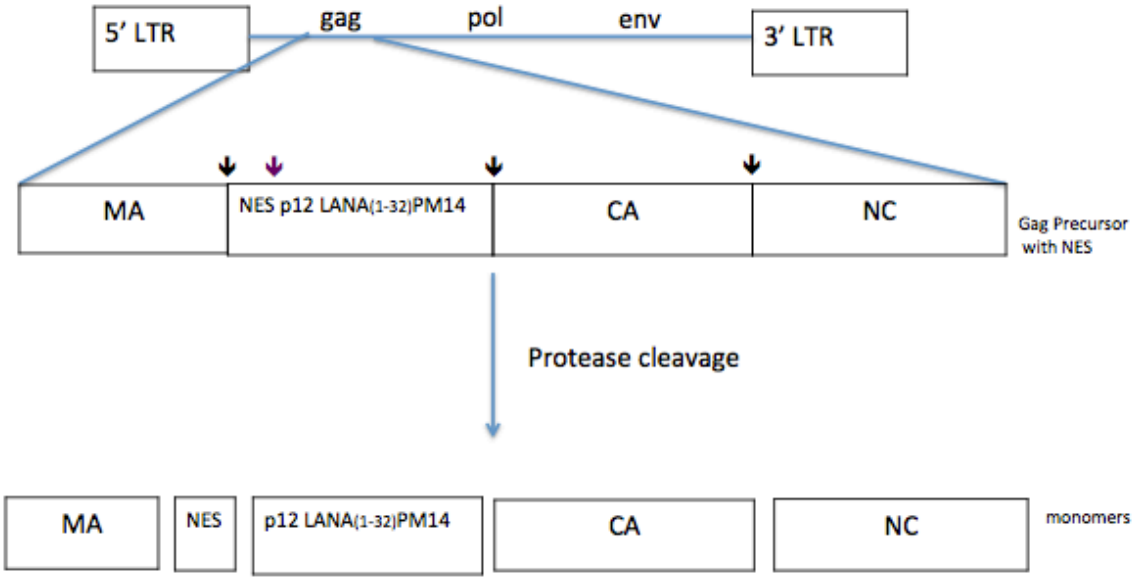


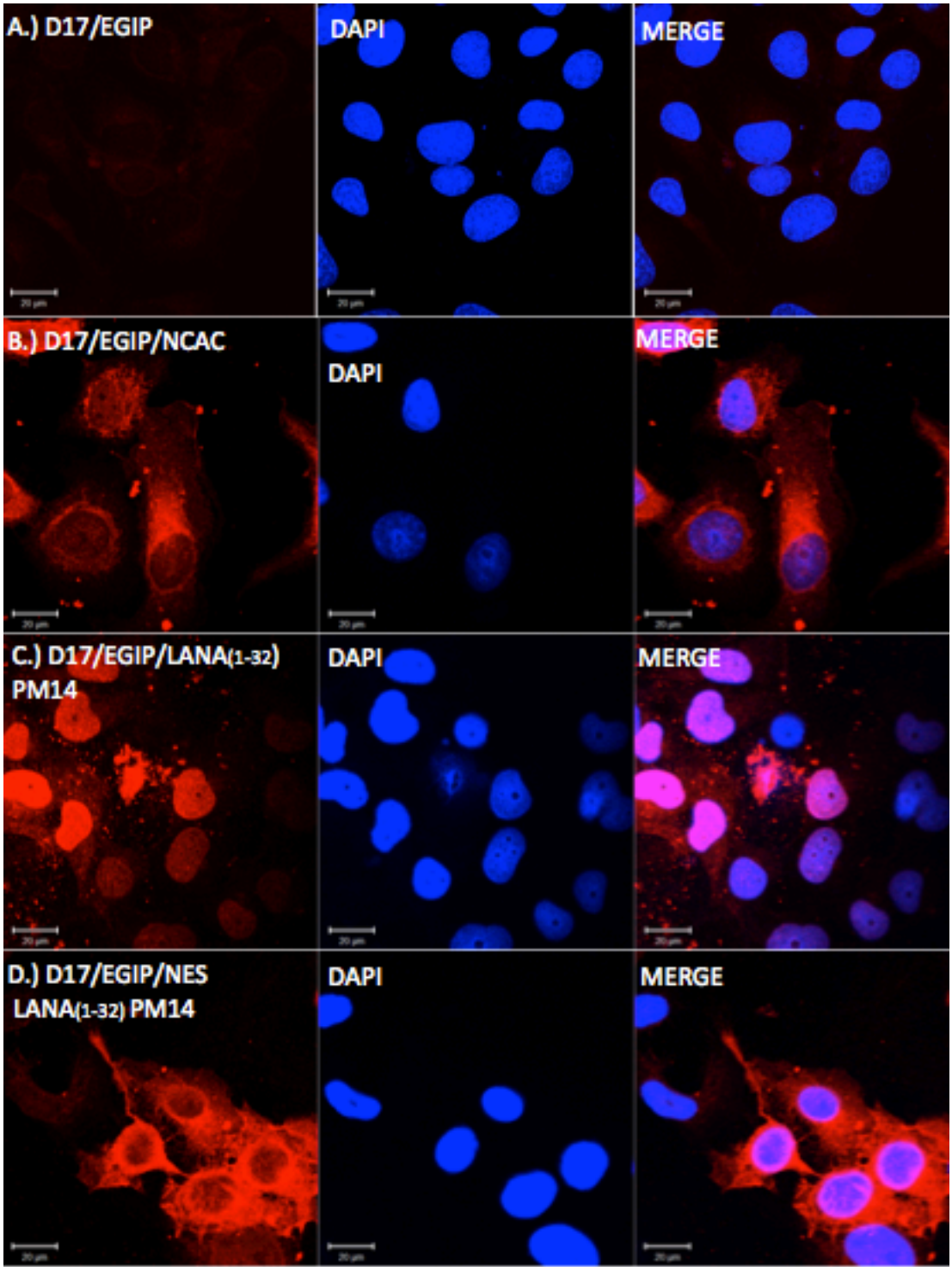
Figure 5: Schematic representation of Gag processing after protease cleavage. The new protease cleavage site is indicated by the purple arrow. After protease cleavage, the NES separates from p12 LANA (1-32) PM14.

3.2 Localization of Gag protein in viral producer cell lines expressing NCAC P12 LANA(1-32) PM14 in the nucleus, whereas those expressing NCAC NES p12 LANA(1-32) PM14 or NCAC NES PR P12 LANA(1-32) PM14 inside the cytoplasm.

It was previously shown that while alternative tethering domains could be inserted into p12 to rescue p12-PM14 mutants, p12 LANA (1-32) showed no RT-activity from supernatants collected over a 40-day period from D17pJET cells transfected with the proviral DNA (Schneider et al., 2013). Stable cells lines of the wildtype (WT) NCA-C-NeoR (Schneider et al., 2012) and the p12-PM14 IN- NeoR mutants were stained for capsid and imaged on a confocal microscope (Figure 6). MLV Gag proteins have not

been found to contain NLS, unlike in HIV-1 (Haffar et al., 2000), and hence Gag precursors are not retained in the nucleus, but exit the nucleus and assemble at the plasma membrane where they ultimately bud off and this is seen in D17/EGIP cells expressing wild type NCA-C-NeoR (Figure 6 B); capsid is localized at the plasma membrane identified in the DAPI-capsid merge. In contrast, Gag encoding LANA (1-32) PM14 is localized in the nucleus (Figure 6 C). The D17/EGIP cell lines with Gag expressing NES LANA (1-32) PM14 and NES PR LANA (1-32) PM14 showed capsid localization in the cytoplasm, hence insertion of the NES or NES PRsite into p12 LANA (1-32) PM14 enables export of the Gag precursor out of the nucleus and into the cytoplasm (Figure 6, panels D and E), counterbalancing the effect of the NLS sequence in MLV p12 LANA (1-32) PM14. The controls D17/EGIP cell lines expressing Gag encoding NES p12 and NES PR p12 show capsid localization at the plasma membrane (Figure 6 F and G respectively), similar to wild type NCA-C-NeoR and therefore, we could minimally infer that there was no gross change in p12 function.

These results indicate that insertion of the NES or NES PRsite into p12 LANA (1-32) PM14 is able to localize Gag precursor which would otherwise be in the nucleus, into the cytoplasm and that the NES sequence does not mislocalize wild type Gag. We then went further to investigate the viral kinetics of the NES constructs.


A.) D17/EGIP
DAPI
MERGE
B.) D17/EGIP/NCAC
DAPI
MERGE
C.) D17/EGIP/LANA(1-32) PM14
DAPI
MERGE
D.) D17/EGIP/NES LANA(1-32) PM14
DAPI
MERGE

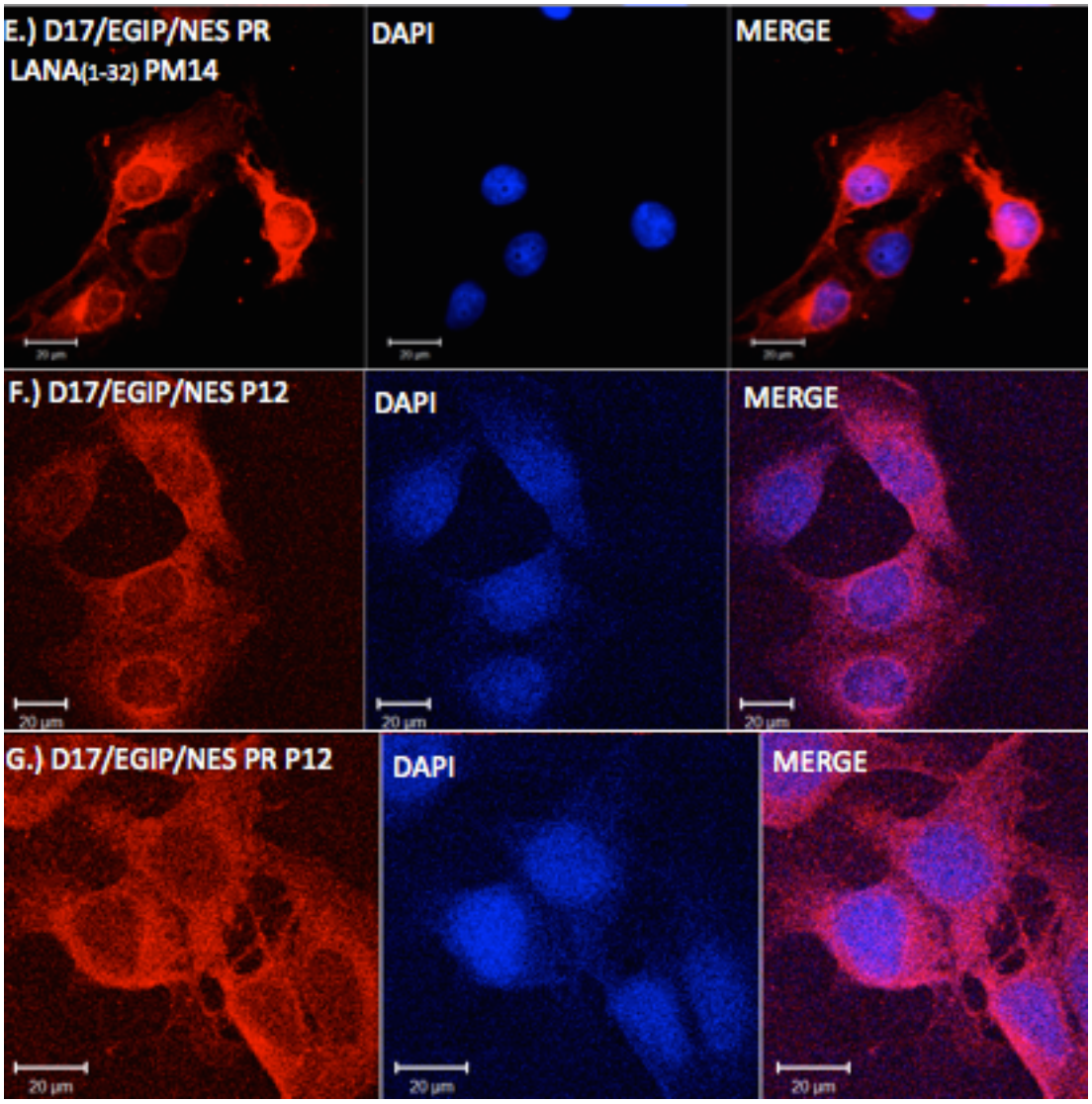


Figure 6: Confocal microscopy pictures of DI7/EGIP viral producer cell lines transfected with each of the NeoR constructs and selected for puromycin and neomycin resistance. The cells were analyzed by immunofluoresce using anti-CA (Red). DAPI staining of the nuclei are in blue. MERGE indicates Capid-DAPI overlap. Panel A shows control DI7/EGIP cell line without any of the NeoR construct. Panel B is wild type MLV. Panels C, D, and E are the producer cells lines expressing Gag p12 LANA (1-32) PM14 with and without the NES insert. Control cell lines expressing Gag NES p12 AND Gag NES PR P12 are shown in panel F and G respectively. (Scale bars, 20 µm).

3.3 NES fails to rescue virus bearing NES p12 LANA (1-32) PM14 in live viral passage assay

To test whether the NES constructs were replication competent, D17pJET cells were transiently transfected with proviral DNA. Virus production was monitored over 40 days through a reverse transcription (RT) assay (Figure 7). Results show wild type NCA-C (Felkner and Roth, 1999) was positive by day 8, whereas viral constructs encoding p12 LANA(1-32) PM14, NES p12 LANA(1-32) PM14, and NES PR p12 LANA(1-32) PM14 were not viable; no RT activity could be detected in the viral supernant collected and tested over the 40 day period. Controls NCAC NES P12 and NCAC NES PR P12 were RT-positive at about day 12 and day 10 respectively, indicating a delay compared to WT NCAC. The RT-positive profile was statistically significant between WT NCAC and controls NCAC NES P12 and NCAC NES PR P12; WT NCA-C was RT-positive 2 days faster than NCAC NES PR P12 and 4 days faster than NCAC NES P12. This indicates that the presence of the NES did not grossly block the infectivity of MLV. While NCAC NES PR P12 appeared to have a slightly better RT-positive profile compared to NCAC NES P12, there was no statistically significant difference between the two viral constructs. None of the mutant P12 constructs encoding LANA(1-32), nor the controls NCAC NES P12 and NCAC NES PR P12, showed any viral generated reversion mutations after day 40 (mutations that restore original phenotype), confirmed by sequencing analysis.

Inserting the NES into MLV p12 LANA(1-32) PM14 did not rescue it. Hence, virus bearing p12 LANA(1-32) PM14 is not compromised due to the interference of the NLS, since we were able to export the Gag precursor out of the nucleus as shown in our

producer cell lines expressing p12 LANA(1-32) PM14 with the NES, but may be compromised due to its tight tethering to mitotic chromosomes. To help better understand this, we tested the ability of the NES in MLV p12 LANA(1-32) PM14 infect other cells.

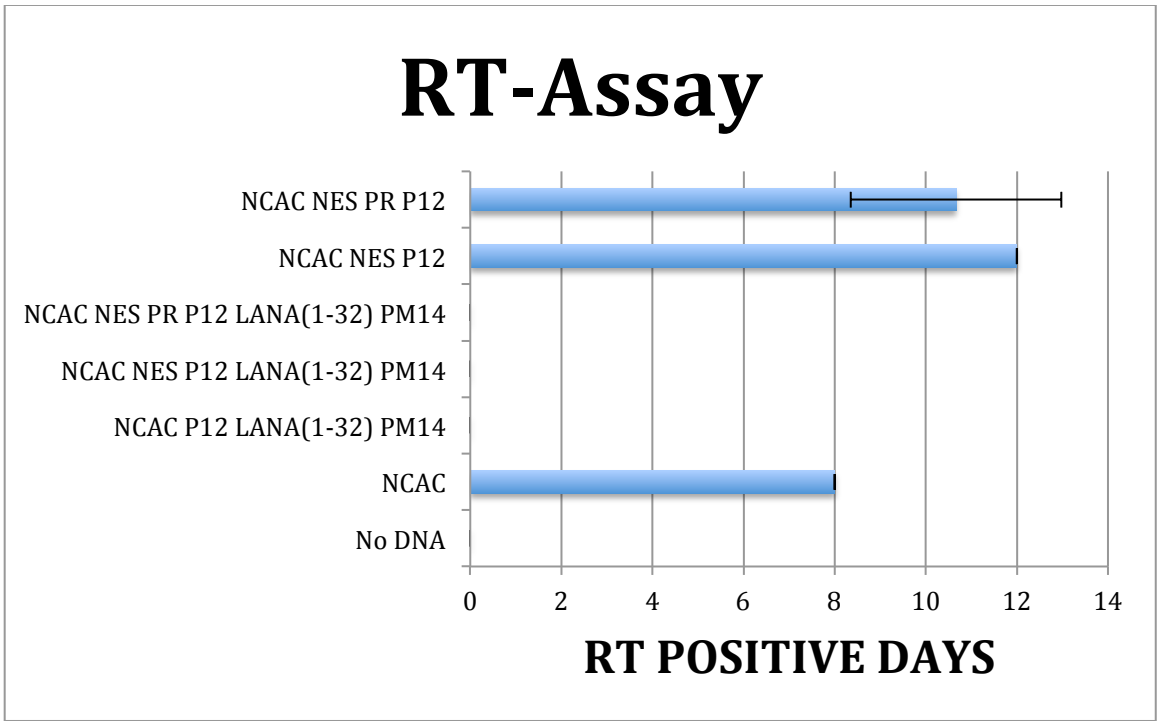


Figure 7: Reverse transcriptase (RT) assay of viral supernatants from DEAE-dextran transfected D17pJET cells (n=3). DNA sequencing of NCAC NES P12 and NCAC NES PR P12 did not indicate reversions after day 40.

3.4 Virus bearing p12-LANA(1-32)-PM14 with NES shows a 10-fold decrease in viral titer

Supernatants from 293T cells transiently transfected with 3µg of DNA (1 µg each of lacZ plasmid, VSG-V, and the respective viral constructs) using FuGENE 6 reagent was used to infect canine D17 cells using 5 ng of capsid. The infection was normalized by a modified ELISA assay (Wu et al., 2013). Results show NCAC P12 LANA(1-32) to have viral titer 100-fold lower than WT NCAC, whereas virus bearing p12 LANA(1-32)

with NES was 10-fold lower and these results were statistically significant (Figure 8). In contrast, there was no statistically significant difference between WT NCAC and the controls NCAC NES P12 and NCAC NES PR P12 showing they have similar viral titer. Our IN mutant (IN-) virus control did not transfer lacZ.

These results show that the NES overcomes the problem of Gag export in p12-LANA$_{(1-32)}$-PM14 to give a 10-fold increase in infectivity compared to NCAC p12-LANA$_{(1-32)}$-PM14, but with a 10-fold decrease in infectivity compared to WT NCAC, but cannot give detectable levels of reverse transcriptase activity (Figure 7).

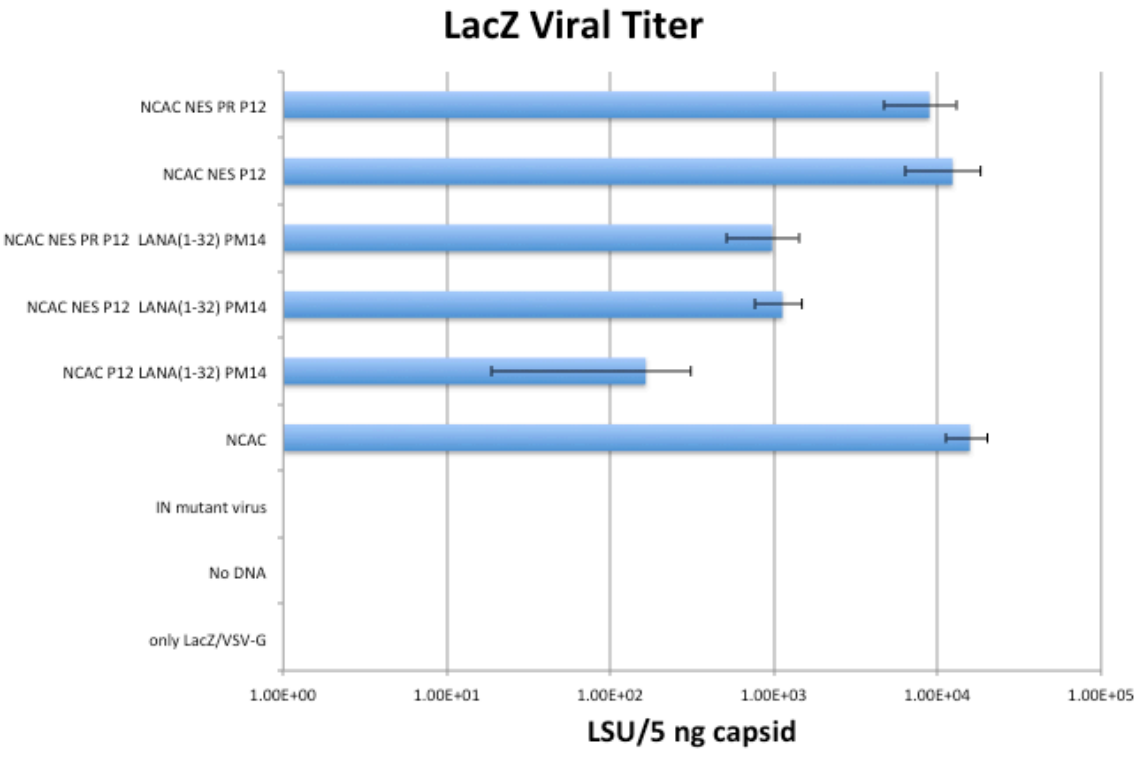
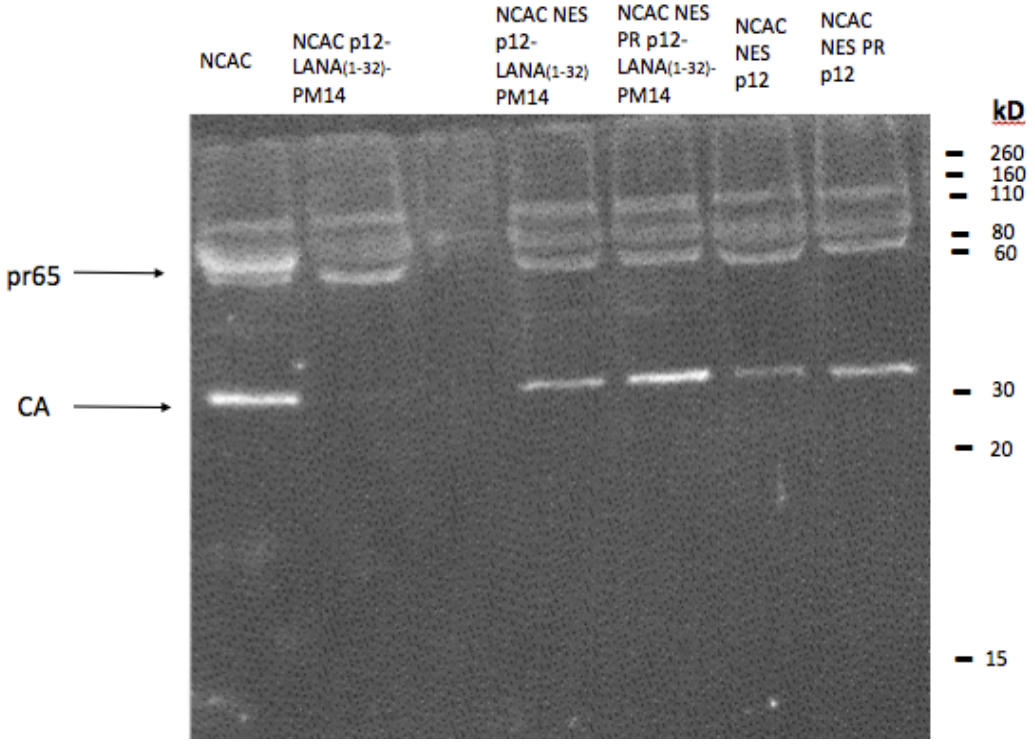


Figure 8: LacZ viral titer assay. Single round infection of canine D17 cells infected with 5 ng capsid from viral supernatants transfected with lacZ plasmid, VSG-V, and proviral DNA. Viral supernatant used for the infection was normalized by an ELISA assay (n=3). Quantification based on lacZ units (LSU) per 5ng capsid.

3.5 Capsid detection in all p12 LANA(1-32) PM14 constructs

To demonstrate capsid release across all viral constructs, 1 ml of viral supernatants from the viral titering assay (i.e. transient transfections on 293T cells with proviral DNA) was pelleted and ran on a 10% SDS PAG and transferred onto a PDVF membrane. Subsequent antibodies were used to probe for capsid (anti-p30 81S263 primary antibody, followed by bovine anti-goat secondary antibody). Results show relatively equal amounts of capsid release in all viral constructs except from NCAC P12 LANA(1-32) PM14 (Figure 9A), consistent with the images from D17/EGIP/LANA (1-32) PM14 viral producer cell line showing Gag precursor localization in the nucleus (Figure 6C). The Western blot shows mostly Gag precursor from NCAC P12 LANA(1-32) PM14 (pr65), but insertion of the NES into p12 LANA(1-32) PM14 overcomes the problem of Gag export, also consistent with our confocal microscopy images (Figure 6, panels D and E). Figure 9B shows the same PVDF blot in 9A reprobed with anti-p12 serum. Here, you can see the p12 bands shift up with the inserted LANA(1-32). You also see precursor MA-p12 (27kDa) bands overlapping the previously blotted capsid bands, and again shifting up with the inserted LANA(1-32) and LANA(1-32) with the NES sequence (pre-cleavage).

A.



B.

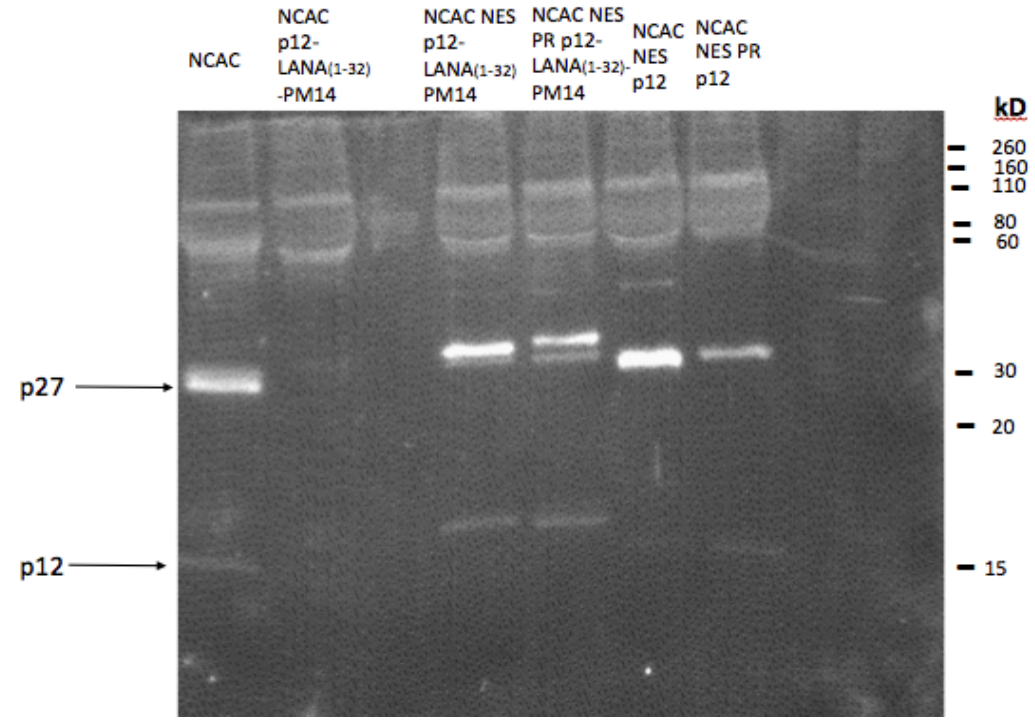


Figure 9: Quantification of viral particles by Western blotting of CA. Viral supernatants were pelleted from 293T cells transfected with 3 µg of DNA (lacZ plasmid, VSG-V, and the respective viral constructs) and loaded onto 10% SDS-page gel and transferred onto a membrane where it was probed first for capsid(A) and then for p12(B).

3.6 Reverse transcriptional products found in virus bearing p12 LANA(1-32) PM14 with the NES is within 2-fold difference of WT

To determine whether the viral titer defect was at the level of reverse transcription, transient transfections of the p12 mutants onto 293T cells were performed (Materials and Methods). Infections were normalized by qPCR onto Te671 cells. 24-hours post infection, the infected cells were trypsinized and DNA was extracted from the cells. Minus strand strong stop (MSSS) intermediates (early RT products), the first products of viral reverse transcription, were assayed and normalized to the RPPH1 housekeeping gene. The results show minus strand strong stop intermediates at levels comparable to wild type NCAC across the viral constructs with the NES, but NCAC p12 LANA(1-32) PM14 is half as much.

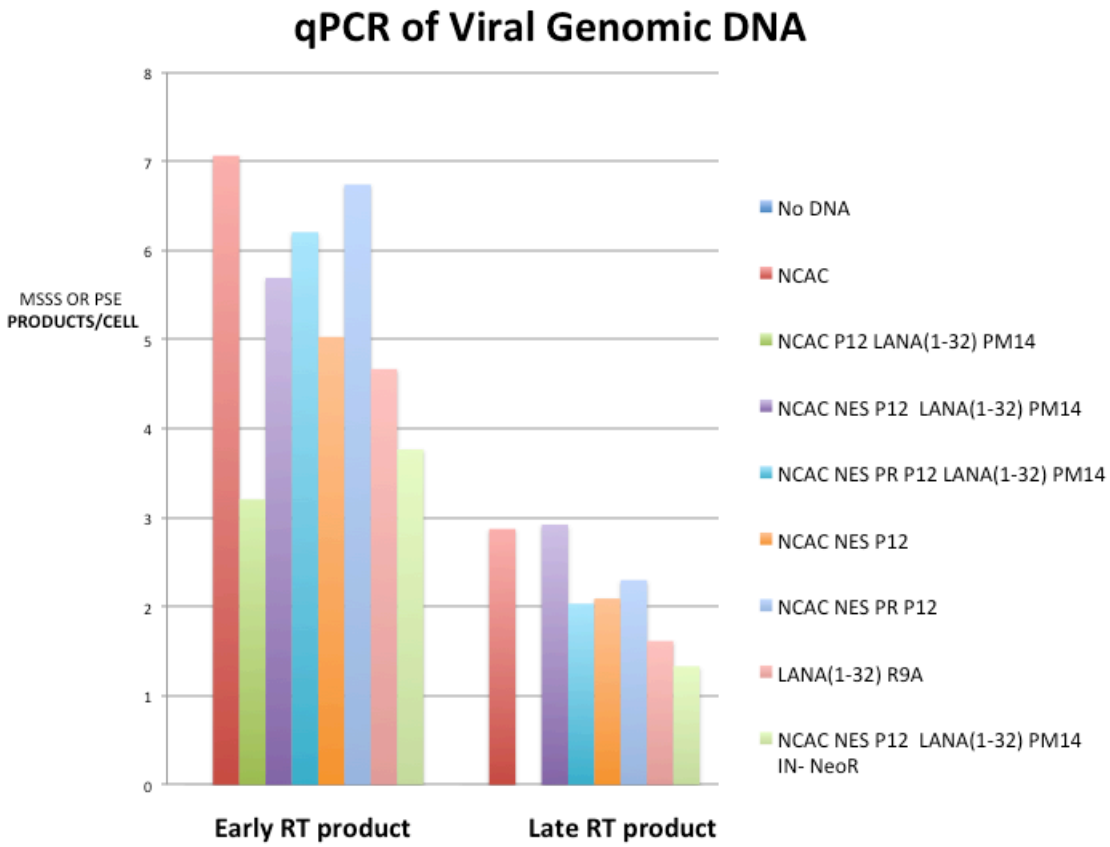


Figure 10: qPCR on gDNA for Early RT product (MSSS) and Late RT product (PSE) replication products (n=1). PSE data missing for NCAC P12 LANA(1-32), because there was not enough genomic DNA to run the reaction.

Chapter 4: DISCUSSION

It was previously shown that the isolate LANA(1-32) PM14 (Schneider et al., 2013) fused to GFP bound tightly to mitotic chromosomes and that insertion of LANA(1-32) within p12 PM14 could not rescue MLV p12-PM14 (Schneider et al., 2013). In this study, we showed that producer cells expressing p12 LANA(1-32) PM14, showed the gag precursor to be nuclear, suggesting that the NLS in LANA(1-32) was retaining the precursor protein in the nucleus. We showed that the addition of a nuclear export signal to this viral construct that localized the gag precursor in the nucleus exported the precursor protein out of the nucleus into the cytoplasm. Hence, the NES enables p12 LANA(1-32) PM14 to overcome the problem of Gag exportation. When the replication kinetics of live virus bearing p12 LANA(1-32) PM14 with the NES was studied, the DEAE Dextran transfections showed the virus was not viable; reverse transcriptase activity could not be detected in any of the Gag constructs encoding p12 LANA(1-32) PM14 and hence, the NES could not rescue p12 LANA(1-32) PM14 in the transient expression assay. Our qPCR data using DNA extracted from cells from single round infections gave MSSS products comparable to wild type virus. Hence, there is reverse transcription with infection. Our viral titer assay showed a 10-fold increase in infection with the NES constructs compared to MLV p12 LANA(1-32) PM14 and this was due to the exportation of the virus out of the nucleus. In summary, MLV p12 LANA(1-32) PM14 with NES enables Gag release from the nucleus into the cytoplasm, and the virus we collected from transient transfections to do an infection gave us a 10-fold decrease in infectivity and reverse transcription comparable to WT, but have the virus dead by dextran (shows no reverse transcriptase activity). From this, it may be gathered that original deduction put forth in Schneider et

al., 2013 that the strong viral tethering to mitotic chromosomes is deleterious to MLV infection and that the block in the viral lifecycle is post-replication and before integration.

Chapter 5: REFERENCES

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