INVESTIGATION OF THE EFFECTS OF THE HIV-1 TAT GENE ON THE EXPRESSION OF SECRETORY LEUCOCYTE PROTEASE INHIBITOR IN PRIMATE CELL LINES

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by

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ABSTRACT

INVESTIGATION OF THE EFFECTS OF THE HIV-1 TAT GENE ON THE EXPRESSION OF SECRETORY LEUCOCYTE PROTEASE INHIBITOR IN PRIMATE CELL LINES

Old world monkey species including African green monkey (AGM) are resistant to HIV-1. Although the virus can gain entry into the susceptible cells of these monkeys, virus replication is blocked prior to viral genome integration by the restriction factors. HIV-1 Tat are the first viral protein that are produced after viral genome integration and they are essential for the production of other viral proteins. According to our preliminary results, AGM CV-1 cells that are known to be resistant to HIV-1 were transfected with HIV-1 regulatory tat. Then, protein profiles of Tat and empty vector expressing AGM CV-1 were compared by 2D-PAGE and that way observed to be induced in cells expressivity tat was identified to be SLPI by mass spectrometry analysis. Based on our literature reviews, SLPI is found to be an anti-bacterial, anti-fungal and anti-inflammatory protein. SLPI also has an extracellular anti-HIV-1 effect.

Our study aims to measure SLPI expression level in AGM and human cells in presence of HIV-1 tat and to research effect of SLPI on NF-kB and HIV LTR promoter. In our research, expression level of SLPI gene is measured by QRT-PCR method. SLPI protein was screened by Western Blot method. SLPI's effect on NF-kB and HIV-LTR promoter was researched through the luciferase experiment. While SLPI gene shows increase in AGM cells in presence of HIV-1 tat, no such increase was observed in human cells. Furthermore, it has been shown that SLPI gene decreases luciferase expression dependent on NF-kB promoter and HIV-LTR. In other words, SLPI suppresses NF-kB promoter and HIV-LTR.

ÖZET

HIV-1 TAT GENİNİN, PRİMAT HÜCRELERİNDEKİ SALGISAL LEKOSİT PROTEAZ İNHİBİTÖRÜNÜN İFADELENMESİ ÜZERİNE OLAN ETKİSİNİN İNCELENMESİ

Afrika Yeşil Maymunu (AYM)'nun da içinde bulunduğu eski dünya maymunu türleri HIV-1'e karşı dirençlidirler. HIV-1 virüsü, bu maymunların hücrelerine girebilmesine rağmen hücre içinde viral genomun entegrasyonundan önceki aşamalarda bloke olmaktadır. HIV-1 Tat ve Rev proteinleri, HIV-1 viral genomunun hücreye entegrasyondan sonra üretilen ilk viral proteinlerdir ve diğer viral proteinlerin üretilmesi için mutlaka gereklidir. Yaptığımız ön çalışmada, HIV-1 enfeksiyonuna karşı dirençli olduğu bilinen AYM CV-1 hücre hatlarına HIV-1 gen ekspresyonunu yönlendiren tat ve rev genlerini aktardık. Daha sonra, HIV-1 Tat ve Rev proteinlerini üreten ve sadece boş vektör içeren AYM CV-1 hücre hatlarından elde ettiğimiz proteinlerin profillerini karşılaştırdık. Protein spotlarından birinin analizi sonucunda, HIV-1 Tat ve Rev proteinlerini eksprese eden hücre hattında SLPI (sekretör lökosit proteaz inhibitörü) protein ekspresyonunda artış tespit ettik. Literatür taramalarımız sonucunda, SLPI'nın antibakteriyel, anti-fungal ve anti-inflamatuar etkilerinin yanı sıra hücre dışı anti -HIV-1 etkisinin (virüsün hücre içerisine girmeden etkisiz hale getirilmesi durumu) de olduğunu saptadık.

Çalışmamızda HIV-1 tat varlığında AGM ve insan hücrelerindeki SLPI ekspresyon seviyesinin ölçülmesi, SLPI'nin NF-kB ve HIV LTR promoter üzerine etkisinin araştırılması amaçlanmıştır. Araştırmamızda SLPI geninin ekpresyon seviyesi QRT-PCR yöntemi ile ölçülmüştür. SLPI proteini Western Blot yöntemi ile görüntülenmiştir. Lusiferaz deneyi ile SLPI'nin NF-kB ve HIV-LTR promotörü üzerine etkisi araştırılmıştır. SLPI geninin HIV-1 tat varlığında AGM hücrelerinden artış gösteririken, insan hücrelelerinde böyle bir artış gözlenmemiştir. Ayrıca SLPI geninin NF-kB promotörüne ve HIV-LTR bağlı lusiferaz ekspresyonu azalttığı gösterilmiştir. Diğer bir ifadeyle SLPI nin NF-kB promotörünü ve HIV-LTR' ı baskılamaktadır.

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CHAPTER 1

INTRODUCTION

1.1. Human Immunodeficiency Virus (HIV)

HIV, which belongs to the retroviridae virus family, is the causative agent of the Acquired Immunodeficiency Syndrome (AIDS) (Miller, et al. 2000). There two types of HIV that is named as HIV-1 and HIV-2 (Cowan, et al. 2002, Requejo 2006).

SIV (infection of macaques) and FIV (infection of cats) have same characteristics. HIV-1 was shown to be derived by zoonosis from West African chimpanzees infected with a very similar virus known as SIVcpz (Pandrea, et al. 2008, Papathanasopoulos, et al. 2003, Hahn, et al. 2000, Holmes 2001, Heeney, et al. 2006, Besnier, et al. 2002). HIV-2, which also can cause AIDS in humans was shown to be derived by zoonosis from sooty mangabey monkeys infected with SIVsmm (Sharp, et al. 2011). However, both the prevalence and infectivity of HIV-2 is lower compared to HIV-1. HIV-1 strains can be divided into three distinct groups. Groups N and O viruses are rare and the vast majority of HIV infections are caused by the group M viruses (Heeney, et al. 2006, Sharp, et al. 2005, Requejo 2006)

HIV and AIDS affect the entire world. There are approximately 40 million people worldwide, living with HIV and AIDS. Over one million People died from HIV related causes globally in 2014. The number of newly infected people exceeded two million. Sub-Saharan Africa is the most affected region, with 25 million people living with HIV in 2014. AIDS patients are treated with combinations of antiretroviral drugs which keep viral replication under control. However, the virus is never eliminated from the patients. There is also the risk of the generation of drug resistant HIV strains. Thus, currently there is no effective cure for the HIV infection (World Health Organization 2014).

1.1.1. Structure and Genome of HIV

HIV is a complex retrovirus. Its genome consists of two linear, positive-sense approx. 11 kb single stranded RNA (Cullen 1991, Holmes 2001). HIV genome contains not only *gag, po*l and *env* genes which are common to all retrovirus, but also six additional genes, which are *tat, rev, nef, vif, vpr* and *vpu (*Romani, et al. 2010, Kingsman 1996). Tat and Rev are the regulatory proteins which regulate the transcription and expression of other viral genes. Nef, Vif, Vpr and Vpu proteins are called as accessory proteins because they are not required during viral infection of tissue culture (Kingsman 1996). *gag* and *pol* encodes viral coat proteins and enzymes respectively. The *env* gene codes for the viral envelope glycoprotein which is responsible for the binding of the virus to its host's cellular receptor (Vicenzi and Poli, 2013, Varmus 1988, Cullen 1992, Trkola 2004, Freed 2001, Morrow, et al. 1994).

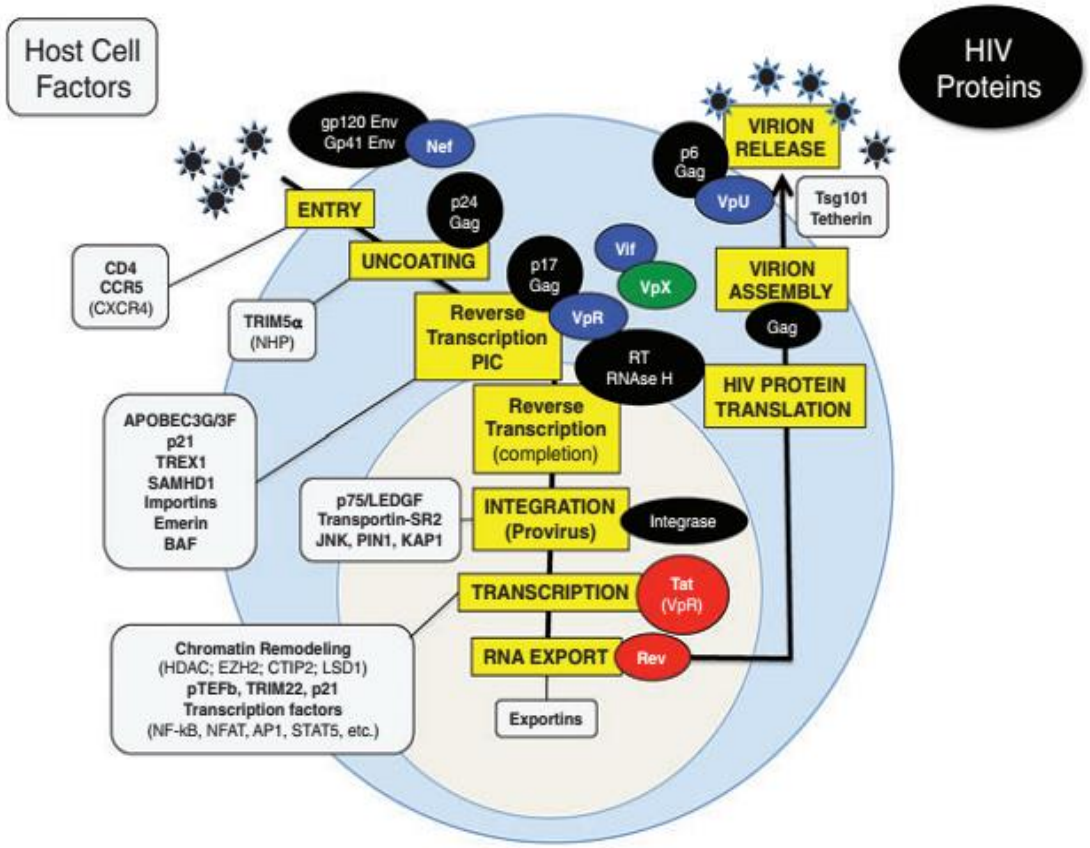

Figure 1.1. HIV Proteins at Viral Life Cycle.
(Source: Vicenzi and Poli, 2013)

HIV genome contains two Long Terminal Repeat (LTR) regions located at both ends of the genome. The LTR which contains the viral promoter is approximately 640 base pairs (bp) in length and is separated into three regions, similar to other retroviral LTRs. Those regions are U3, R and U5. The U3 region has been further subdivided according to transcription factor binding sites which are important for viral gene expression. These sites include negative regulatory elements, NF-kB, Sp1 and TATA box. R region codes the trans-activation response (TAR) region, which is important for viral gene transcription (Krebs, et al. 2005).

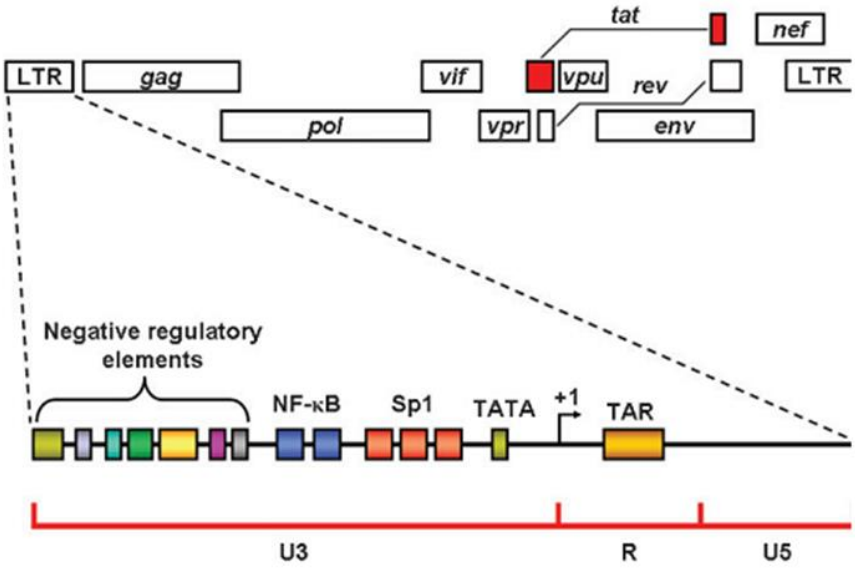

Figure 1.2: Structure of HIV Genome.
(Source: Romani, et al. 2010)

1.1.2. Viral Life Cycle of HIV-1

Life cycle of HIV-1 starts with the infection of a new target cell, as the result of interaction between the target cell's surface receptors (CD4 receptor and a co-receptor CCR5 or CXCR4) (Dimitrov, et al. 1998, Klimas, et al. 2008, Holmes 2001). Following the viral entry, the positive single strand viral RNA is converted into the double strand viral DNA by viral reverse transcriptase. Thereafter, the viral DNA creates a pre-integration complex (PIC) and it is imported to the host cell's nucleus. The viral DNA is then integrated into the host cell's genome by the viral integrase. The integrated viral genome is called as the provirus. Following the integration phase, this provirus remains

latent for many years unless the infected cell produces the required transcription factors for HIV gene expression (Potter, et al. 2007, Klimas, et al. 2008). If infected cell is activated, started to synthesize viral proteins by transcription which is regulated from both host cell and viral genes. In this process, RNA is transcribed from DNA which is called viral mRNA and transported from host cell nucleus to cytoplasm. Following this stage, new viral proteins are produced from viral mRNA. This process is called translation (Klimas, et al. 2008). New viral proteins and RNAs go to cell surface of host cell and assemble into immature form, which is noninfectious (Briggs, et al. 2006). Virus then budding of the host cell membrane and itself out of the cell. New virus releases viral proteases, which cut of the long protein chain. The smaller new viral proteins forms mature virus, which is infectious (Sundquist, et al. 2012).

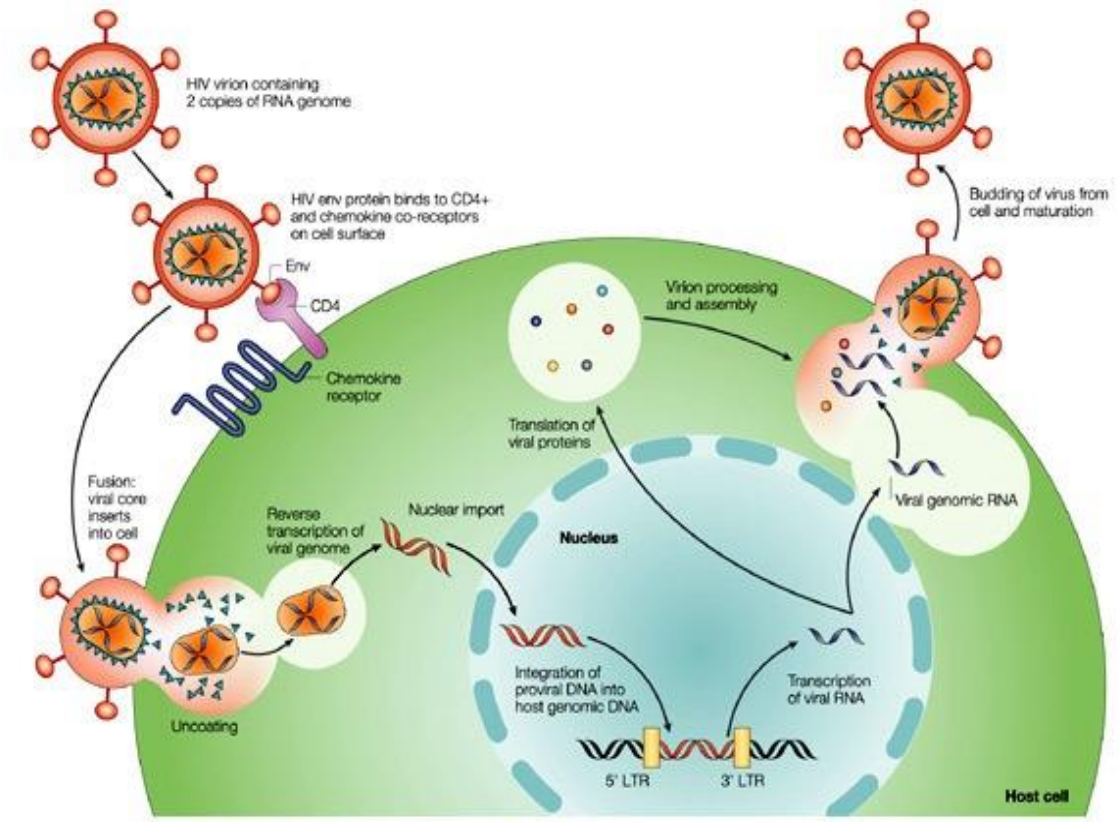

Figure 1.3. HIV Replication Life Cycle.
(Source: Rambaut, et al. 2004)

Viral pre-messenger RNA is initially synthesized from HIV genome (Bohne, et al. 2005, Wu 2004, Nielsen, et al. 2005, Holmes 2001, Klimas, et al. 2008). Some of viral pre-mRNAs are completely spliced, some of viral mRNAs incompletely spliced

and other viral mRNAs are unspliced, which are exported to host cell cytoplasm mediated by the viral regulatory protein Rev (Stoltzfus, et al. 2006, Cochrane, et al. 2006, Cullen 2003). Spliced HIV-1 mRNAs can be divided two groups, Rev-dependent 4-kb in length and Rev independent 1.8 kb in length. Env/Vpu, Vif, and Vpr are encoded the Rev dependent mRNAs (Dayton, et al. 2004, Schwartz, et al. 1991). Viral regulatory proteins Tat, Rev and Nef are encoded by the Rev independent mRNAs. While Rev dependent mRNAs are transported to cytoplasm mediated by Rev/CRM1 pathway, Rev independent mRNAs are transported to cytoplasm mediated by export factor NXF1 (Araya, et al. 2015)These are translated in the early stage of viral life cycle (Tazi, et al. 2010, Kim, et al. 1989, Michael et al. 1991, Munis, et al. 1992).

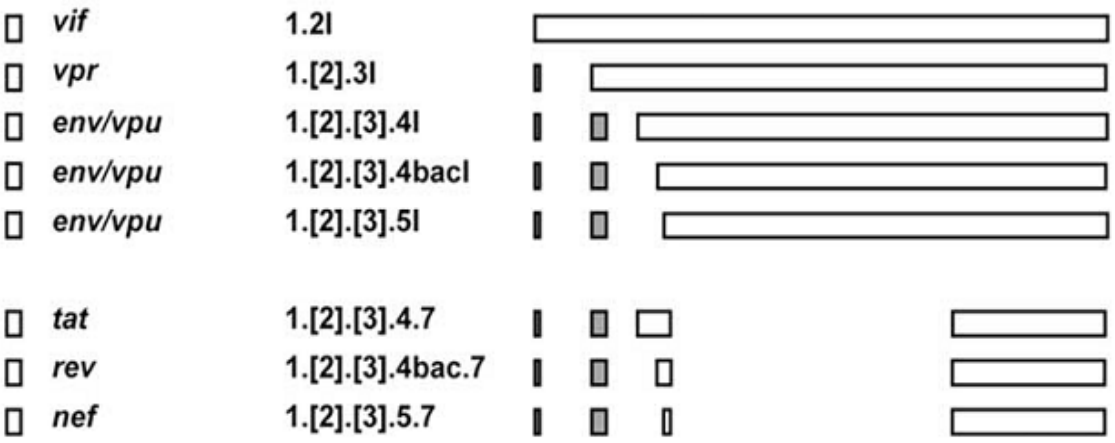

Figure 1.4. Spliced HIV-1 mRNAs.
(Source: Schwartz, et al. 1991)

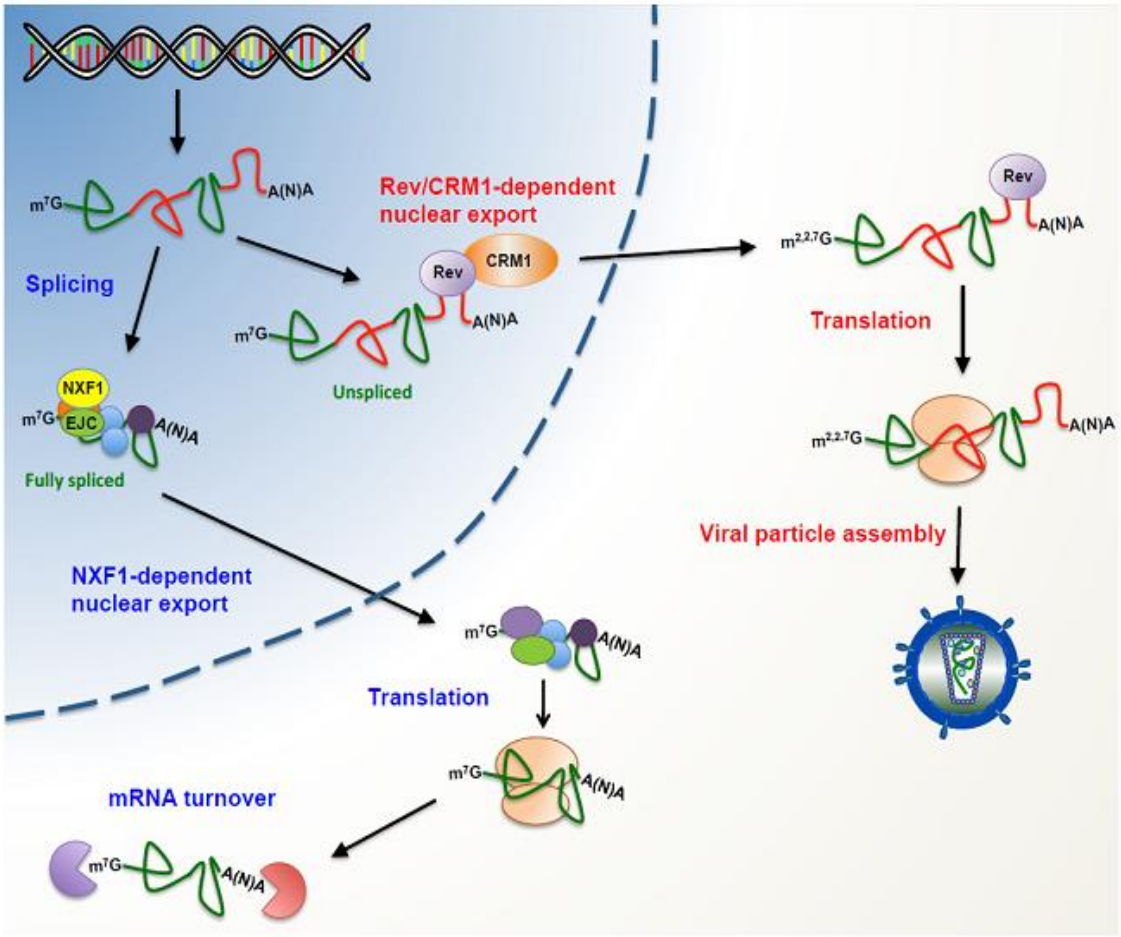

Figure 1.5. Transport of the Viral mRNAs to Host Cell Cytoplasm
(Source: Araya, et al. 2015).

Furthermore, viral mRNAs can be separated into three classes. The multiply spliced mRNAs encode the regulatory proteins such as Tat, Nef and Rev. and the singly spliced mRNA encodes Vpu, Vpr, Vif and Env. And the unspliced mRNA encodes the Gag and Pol (Wu 2004, Pollard and Malim 1998, Frankel and Young 1998, Romanov, et al. 1997, Houzet, et al. 2007; Caputi 2011).

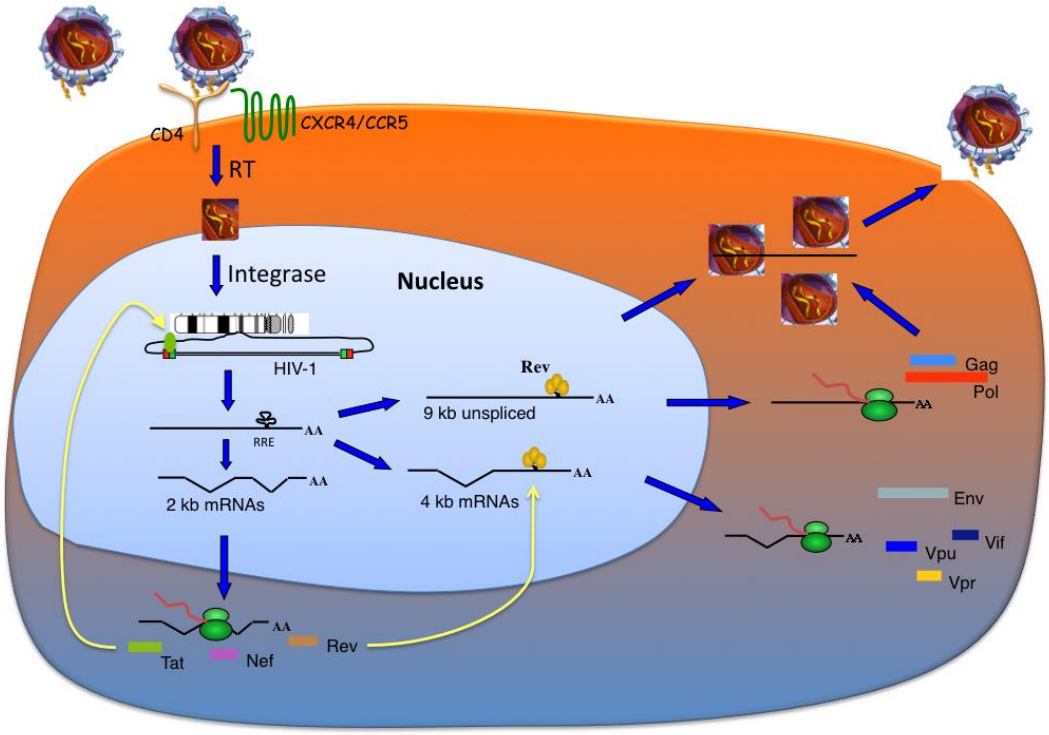

Figure 1.6. Rev Function and Viral mRNA Export.
(Source: Caputi 2011)

1.1.3. Trans Activator Protein (TAT)

Tat protein, synthesized at the early stage of viral life cycle is absolutely required for the HIV-1 transcription (Mayol, et al. 2007, Hooker, et al. 2002, Cullen 1991). Tat is an RNA binding protein with a molecular mass of around 15 kDa (Nekhai and Jeang 2006, Romani, et al. 2010, Pugliese, et al. 2005) and it is located in the cell nucleus (Romani, et al. 2010, Pugliese, et al. 2005).

HIV transcription can be divided into two phases: Early phase (tat-independent) and Late phase (tat-dependent) (Brady and Kashanchi 2005, Marcello, et al. 2001). Tat is an unusual transcription factor because of binding to the cis-acting RNA element TAR (Marcello, et al. 2001, Fittipaldi and Giacca 2005, Pugliese, et al. 2005, Berkhout, et al. 1989, Hetzer, et al. 2005). TAR is located downstream of the transcription initiation sites and Tar RNA is a 59 nucleotide hairpin stem-loop structure (Cullen 1992). Sp1 and NF-kB increase the HIV-1 transcription and play an important role in the tat independent transactivation (Pieper, et al. 2002, Dandekar, et al. 2004). Tat permits activation of an unstable RNA polymerase. RNA polymerase either pauses or

falls off on the HIV template, following the transcription through the TAR region, unless Tat is present in the cells. During transactivation phase, the c-terminal region of RNA polymerase is phosphorylated by TFIIH. Following the clearance of the promoter region, TAR region is transcribed by the RNA polymerase and TAR is synthesized, which acts as a signal for Tat (Gautier, et al. 2009). Thereafter, tat binds to TAR and also binds to the TAK and forms ternary complex. This complex phosphorylates the RNA polymerase and activates the initiation of transcription (Fujinaga, et al. 2004, Charnay, et al. 2009, Karn 1999).

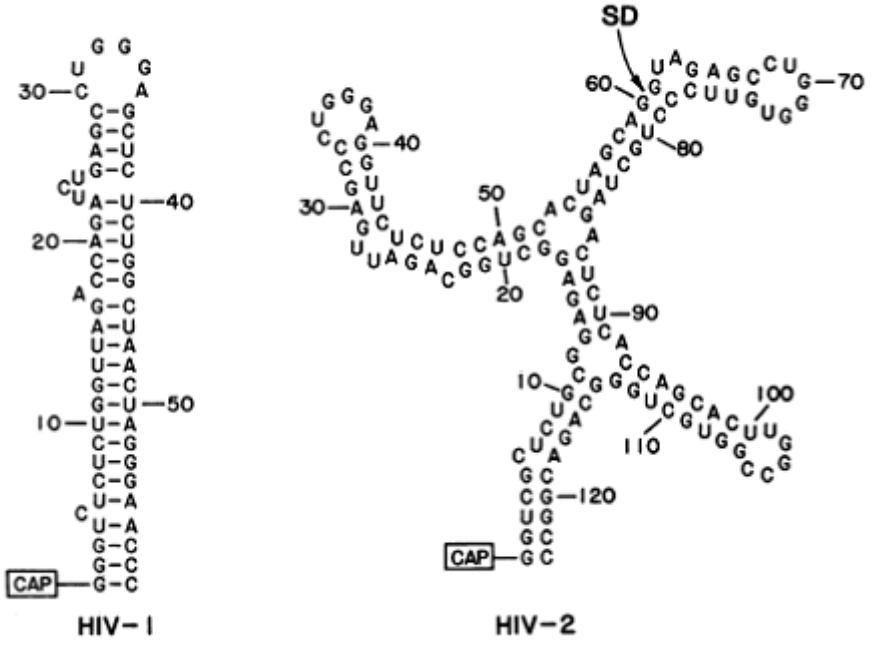

Figure 1.7. RNA Secondary Structure of HIV TARs.
(Source: Cullen 1992)

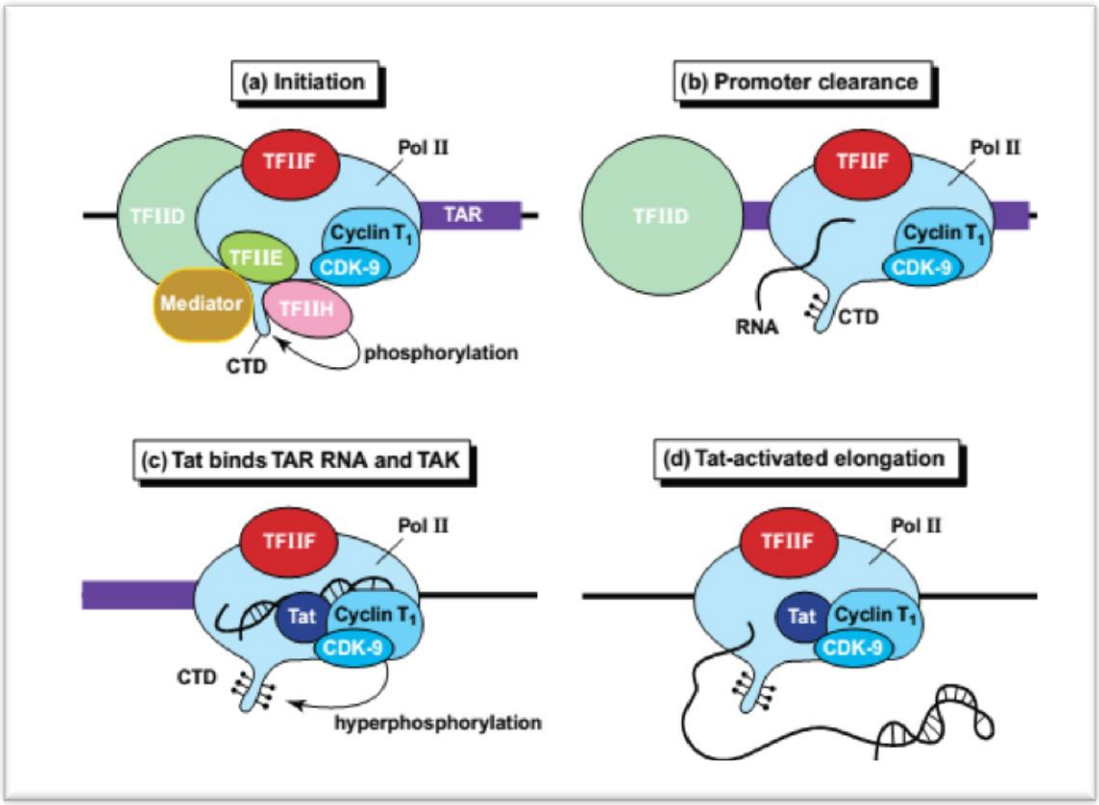

Figure 1.8. Tat Activation of HIV Transcription Initiation.
(Source: Karn 1999)

1.2. Antiretroviral Intrinsic Immunity

Immune system protects the host cell against viral infections and the immune system is divided into two main groups: The innate (natural) immune system and the adaptive immune system (Dranoff 2004).

The innate (natural) immunity consists of various cells and proteins, which are complement proteins, granulocytes, mast cells, macrophages, dendritic and naturel killer cells. These components are always present at the site of infection. However, the adaptive (acquired) immunity consists of B cell, $CD4^+$ and $CD8^+$ T cell. Natural killer cells are both found the innate and adaptive immunity (Dranoff 2004). The recent studies show that there is a specific form of innate immunity, which is the intrinsic immunity. Intrinsic immunity is a very important defense system against many viral infections that targets various steps in the viral life cycle (Emerman 2006, Mangeat and Trono 2005, Goff 2004; Goldschmidt, et al. 2008).

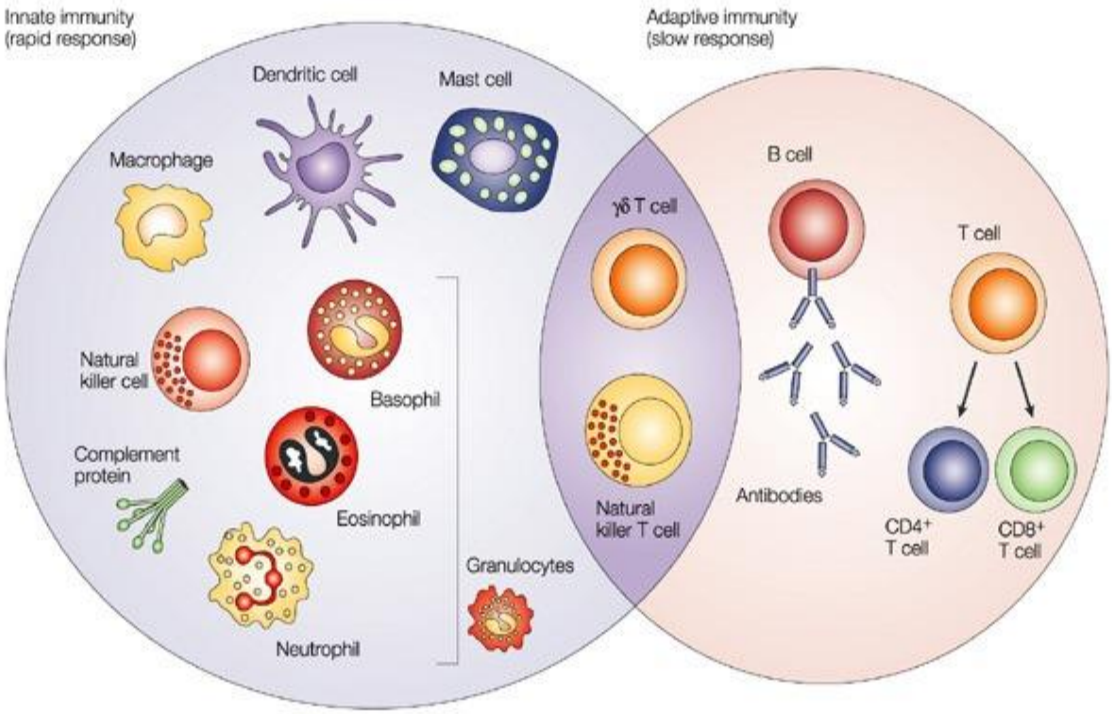

Figure 1.9. The Innate and Adaptive Immunity Components.
(Source: Dranoff 2004)

1.3. Retroviral Restriction Factors

Retroviral restriction factors are intracellular proteins and/or components of the intrinsic immunity evolved to inhibit retroviral infections at different steps of viral life cycle (Greene, et al. 2008, Towers and Goff 2003, Bishop, et al. 2006, Bannert, et al. 2006, Best, et al. 1996, Kaiser, et al. 2007, Goff 2004). All restriction factors and their targets are shown in Table 1.1.

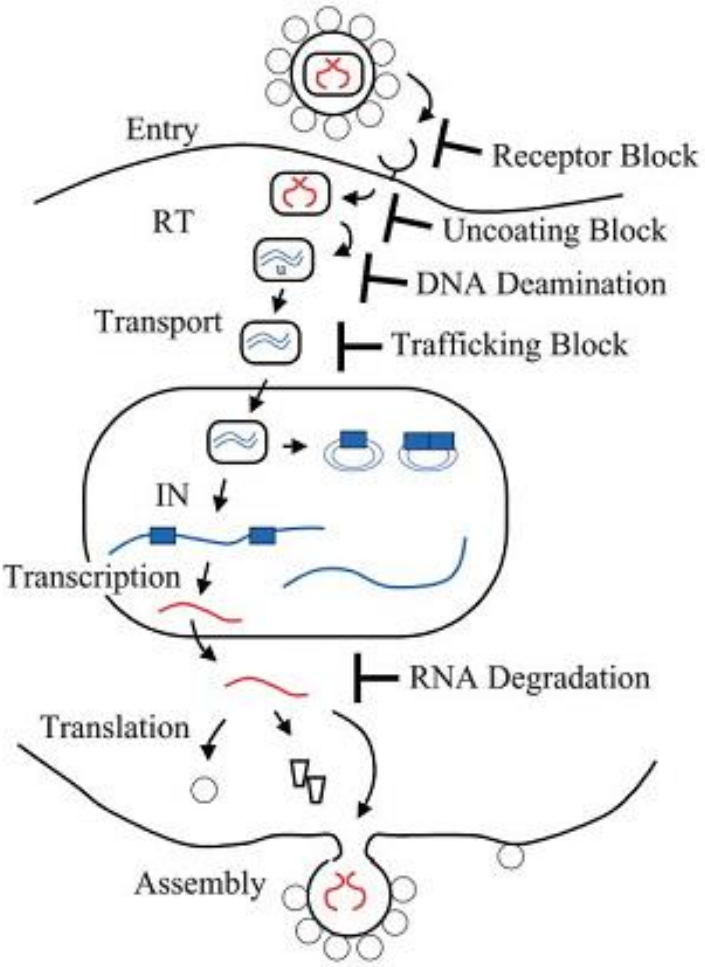

Figure 1.10. Retroviral Restriction Factors Acting at Different Steps of the Viral Life Cycle. (Source: Goff 2004)

1.3.1. Receptor Block/Fv4

The Fv4 gene was identified in laboratory mice, which was shown to encode an envelope (Env) protein, whose putative receptor-binding domain resembles that of the ecotropic murine leukemia virus MuLV Env protein (Wolf, et al. 2008, Taylor, et al. 2001). The Fv4 mediated restriction is achieved by its binding to the MuLV receptor, preventing MuLV binding to the same receptor (Masuda, et al. 1990).

1.3.2. DNA Deamination/APOBEC

A remarkable restriction system has been identified that targets the *vif* deficient retroviruses after the viral entry. The most important proteins of this system are APOBEC family of proteins that are cytidine deaminases, which convert cytosine to

uracil (Malim, et al. 2009, Hache, et al. 2006, Chiu, et al. 2009, Huthoff, et al. 2005, Aguiar, et al. 2008, Goff 2004, Saito and Akari 2013). The APOBEC proteins inhibit retroviruses by introducing mutations in the nascent retroviral DNA by deamination of cytosine residues. HIV encodes Vif, a small protein that mediates APOBEC degradation (Zhang and Webb 2004, Goila-Gaur, et al. 2008, Goff 2004, Harris, et al. 2004). In addition, APOBEC proteins inhibit viral DNA synthesis by disrupting reverse transcriptase (Wolf, et al. 2008, Esnault, et al. 2006, Malim 2009).

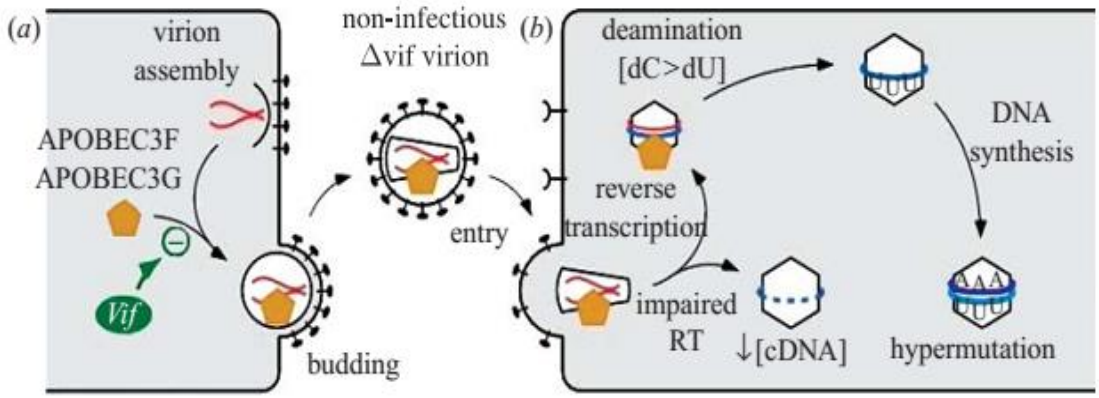

Figure 1.11. Inhibition of HIV Infection by APOBEC Proteins.
(Source: Malim 2009)

1.3.3. Trafficking Block/Fv1

Fv1 is a cytoplasmic protein that is 440 or 459 amino acids in length (Bieniasz 2003) and it is present in some mice strains (Goff 2004). It is known as the first retroviral restriction factor (Towers and Goff 2003). Fv1 blocks retroviral life cycle after the entry of virus but before integration (Wolf, et al. 2008, Bock 2000, Stocking and Kozak 2008, Bieniasz 2003, Goff 2004). Fv1 targets the Murine Leukemia Virus (MLV) capsid protein (Nakayama and Shioda 2010). Furthermore, Fv1 prevents the transport of pre-integration complex of MLV (Sorin, et al. 2006)

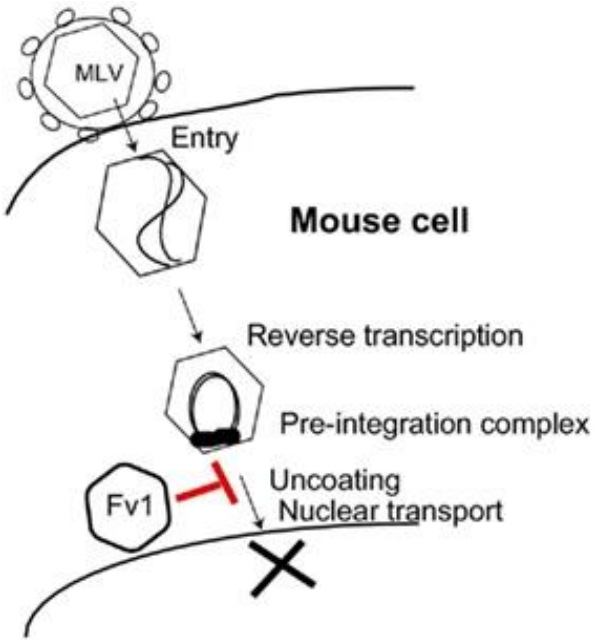

Figure 1.12. Fv1 Restriction Factors Block Retroviral Infection.
(Source: Nakayama and Shioda 2012)

1.3.4. Trim5α

Trim5α is the first block to HIV-1 replication in the cells of Old World Monkeys (OWM) (Nakayama and Shioda 2010, Huthoff and Towers 2008, Streamliu, et al. 2004). Trim5α from OWM binds to the viral capsid. This leads to activation of E3 ligases with the UBC13–UEV1A enzyme complex and free ubiquitin chains are synthesized. These chains induce the expression of nuclear factor kappa B cells (NF-κB) and mitogen-activated protein kinase (MPK) and phosphorylation of TAK-1, resulting in the activation of antiviral innate response (Yamauchi, et al. 2008, Uchil, et al. 2008, Li, et al. 2007, Diaz-Griffero, et al. 2006, Campbell, et al. 2008, Saito and Akari 2013). Therefore, Trim5α is not only HIV-1 replication by binding viral capsid, but also turn on the alarm in the innate immunity in the infected cells. The expression of the OWM version of the Trim5α in human cells inhibits HIV infection (Nisole, et al. 2005, Berthoux, et al. 2005, Silva and Wu 2011).

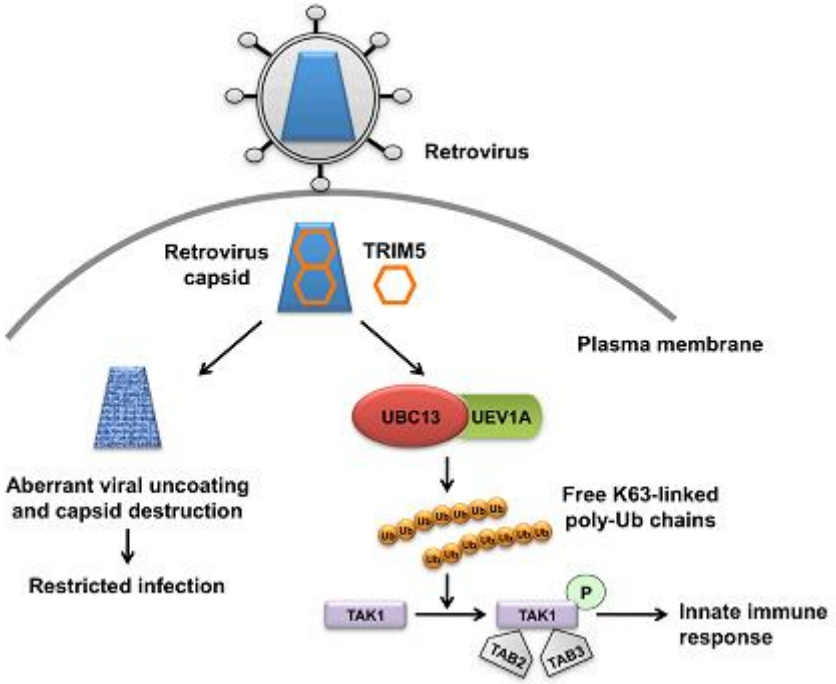

Figure 1.13. Trim5α Binds to Viral Capsid to Block HIV Replication.
(Source: Silva and Wu 2011)

However, it has been shown that TRIM5-alpha is saturable at high doses of the virus. Therefore at high multiplicities of infection some of the HIV-1 particles can escape from the restriction effect of this protein and continue to infect the cell. (Caroline, et al 2002, Nisale, et al. 2005, Daniel, et al. 2008). Neverthless, these monkey species are still resistant to HIV-1 providing an evidence to the presence of protein or proteins other than TRIM5-alpha that interfere with HIV-1 infection at a stage after the integration of the viral genome in those cells (Daniel, et al. 2008, Tatsuo, et al. 2005, Saito and Akari 2013).

Table 1.1. Properties of Dominant Retrovirus Resistant Genes
(Source: Goff 2004)

Species	**Restriction System**	**Time of Block**	**Restricted Viruses**	**Unrestricted Viruses**
Mouse	Fv4	Entry	Ecotropic MuLV	Amphotropic MuLV lentiviruse
Human, Chimpanze	APOBEC3G	Viral DNA formation	Vif-negative HIV-1 SIVAGM EIAV HB	Vif-positive HIV-1
African Green Monkey	APOBEC3G	Viral DNA formation	HIV-1	Vif-positive SIVAGM Vif-negative SIVAGM
Human	APOBEC3F	Viral DNA formation	Vif-negative HIV-1	Vif-positive HIV-1 Vif-positive HIV-2 MuLV
Rhesus macaque	TRIM5	Early postenrty	HIV-1	N-MuLV B-MuLV SIVmac
African Green Monkey	TRIM5 (Lv1/Ref1)	Early postentry	MuLV HIV-1 HIV-2 SIVmac EIAV	B-MuL SIVAGM
Squirrel monkey	TRIM5 (Lv1)	Early postentry	SIVmac	N-MuLV
Owl monkey	TRIM-Cyp	Early postentry	HIV-1	N-MuLV B-MuLV SIVmac
Rodent	ZAP	Viral RNA expression	MuLV HIV-1 alphaviruses	VSV Poliovirus DNA virus

1.4. Host Tropism of HIV-1

Currently there are no animal models of Human immunodeficiency virus 1 (HIV-1) infection since HIV induces AIDS only in humans. Some of the chimpanzees that were experimentally infected with HIV-1 were reported to show AIDS symptoms. However due to the ethical considerations and the endangered status of these animals, they are no longer allowed to be used in such studies (Saito and Akari 2013). Retroviral factors of Trim5 and APOBEC3 protein family inhibit retroviral replication in OWM. Trim5 inhibits retroviruses by binding of the viral capsid, but the APOBEC proteins inhibit retroviruses by introducing mutations in the nascent retroviral DNA by deamination of cytosine residues (Liu, et al. 2005, Silva and Wu 2011, Goff 2004, Harris, et al. 2004). Therefore, the effects of the Trim5 and APOBEC3 family, which described above create strong barrier to HIV-1 replication in OWM (Kazazian, et al. 2006, Saito and Akari 2013). However, experimental infection of OWM was only successful after their immune systems were compromised (Hatziioannou, et al. 2014).

1.5. Preliminary Study

In the previous studies, which were carried out in our lab, initially the main regulator tat gene of HIV-1 was cloned to pBudCE4.1 mammalian expression vector. Thereafter, constructed this pBudCE4.1_tat and empty pBudCE4.1 vectors were transfected to cv1 cell lines, which derived from Africa Green Monkey (AGM) with stable transfection in a proper way, as recommended by the manufacturer. Therefore, cv1-tat cells that express HIV-1 tat gene were constructed (Burcu SENGEZ Thesis 2011). Thereafter, protein isolation was carried out from cv-1 tat cells and cv-1 cell, which involved empty pBudCE4.1 in a proper way, as recommended by the manufacturer. 2D-GEL Electrophoresis was then carried out to detect different expressed proteins. At the end of 2D-GEL experiment, several different protein spots were observed and these spots were analyzed with MALDI-TOF/TOF mass spectrometry. Secretory Leucocyte Protease Inhibitor (SLPI) was found in one of these spots. Previous studies indicate that SLPI has effect of the anti-bacterial, anti-fungal, anti-inflammatory and anti-HIV. Furthermore, SLPI especially inhibits oral

transmission of HIV-1. Therefore, we focus on the effect of SLPI on other AGM and human cells.

1.6. Secretory Leucocyte Protease Inhibitor (SLPI)

Secretory leucocyte protease inhibitor is an 11.7 kDa protein, which is a no glycosylated, highly basic, acid stable, cysteine rich, 107 amino acid and single change amino acid. Furthermore, it belongs to the innate immunity-associated proteins (Doumas, et al. 2005). The tertiary structure of SLPI molecule has a shape similar to a boomerang and its each arms are carrying one domain (Grutter, et al. 1988). Each domain binds to each other with the disulfide bond located between the two cysteine residues and this interaction contributes conformation of SLPI molecule (Hiemstra, et al. 1996, McNeely, et al. 1997, Seemuller, et al. 1986). The SLPI gene involves four exons and three introns and roughly 2.6 kb (Kikuchi, et al. 1998, Stetler, et al. 1986).

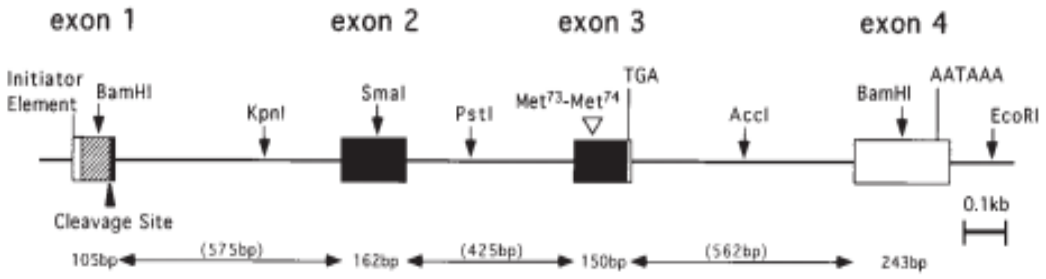

Figure 1.14. Genomic Structure of SLPI.
(Source: Kikuchi, et al. 1998)

SLPI was firstly identified in secretions of patients with chronic obstructive pulmonary disease and cystic fibrosis (Hochstrasser, et al. 1972, Ohlsson, et al. 2001; Tegner 1978). Neutrophils, macrophages, beta-cells of pancreatic islets, epithelial cells investing the renal tubules, acinar cells of parotid and submandibular glands, acinar cells of submucosal glands, and epithelial cells lining mucous membranes of respiratory and alimentary tracts produce SLPI (Abe, et al. 1991, Fahey, et al. 2002, Farquhar, et al. 2002, Jin, et al. 1997, Nystrom, et al. 1999, Shugars 1999, Ohlsson, et al. 2001). SLPI was isolated in various secretions such as seminal fluid, cervical mucus, synovial fluid, breast milk, tears and cerebral fluid as well as secretions from nose and bronchi

(Farquhar, et al. 2002, Franken, et al. 1989, McNeely 1997, Pillay, et al. 2001, Shugars 1999). The SLPI was expressed in the lung, breast, bladder, endometrial, ovarian and colorectal carcinomas and also was found in neurons (Wang, et al. 2003). In addition, the SLPI plays an important role in apoptosis and wound healing (Ashcroft, et al. 2000, Odaka, et al. 2003, Sorensen, et al. 2003).

The SLPI protects the local tissue from harmful effect of inflammation. Serine proteases, which are secreted from leucocyte during inflammation and toxic inflammatory products also it degrades the tissue. SLPI inhibits these proteases such as cathepsin G, elastase, trypsin and chymase (Gipson, et al. 1999, He, et al. 2003, Jin, et al. 1999). In addition, SLPI is shield of tissue against the inflammatory agents. It decreases the response of macrophage and down-regulates NF-kB pathway by preventing inhibiting factor of NF-kB (I-kB) from ubiquitin-proteosome pathway (Hiemstra, et al. 2000, Jin, et al. 1997).

Recent studies indicate that the SLPI has an antibiotic activity that involves bactericidal and antifungal features (Doumas, et al. 2005). In a recent study, Fahey and Wira investigated the production of antibacterial factor(s) by uterus epithelial cell from pre- and postmenopausal periods of women. Staphylococcus aureus and Escherichia coli were efficiently killed in the pre-menopausal women fluid, but not in the postmenopausal women. Thereafter, the SLPI concentration in the premenopausal fluid was significantly higher than the one in the postmenopausal women. SLPI production is associated with the bactericidal activity and with the menstrual status and with the cultivation time. The binding of protease inhibitor to the bacterial mRNA and DNA may cause SLPI mediated bactericidal activity (Hiemstra, et al. 2000, Miller, et al. 1989). SLPI has 50% fungicidal activity against the pathogenic fungi Aspergillus fumigatus and Candida albicans (Tomee, et al. 1997). In addition, SLPI may be an indication for an oral fungal infection, since it is increased in salivary SLPI (Doumas, et al. 2005). It may be an effective therapeutic option in the future treatments and a way for prevention from infection diseases, because of its antimicrobial activity (Tomee, et al. 1998).

The SLPI is the major pro-inhibitor, which is active against the HIV-1 transmission through oral secretions (Challacombe, et al. 2002, Shugars, et al. 1999). SLPI was found as anti-HIV-1 factor in the various innate inhibitory molecules such as virus-specific antibodies mucins and thrombospondins, which were identified and purified from saliva (McNeely, et al. 1995). The anti-HIV-1 activity of SLPI was investigated by several scientist (Doumas, et al. 2005). SLPI significantly suppress infection of the primary adherent monocytes with HIV-1 in human saliva. Only SLPI among the all protein contents of saliva has antiretroviral activity at physiological concentrations (0.1 to 10 g/ml) (McNeely, et al. 1995, McNeely, et al. 1997, Shugars, et al. 1997, Hocini, et al. 2002). Although SLPI has an anti-HIV-1 activity, it has no activity against the other retroviruses, such as murine leukemia virus, human T-lymphotropic virus and simian immunodeficiency virus (Shugars, et al. 1999, Skott, et al. 2002, Wahl, et al. 1997). The anti-HIV-1 activity mechanism of the SLPI is still unclear, but it involves the host cell target rather than binding of viruses (McNeely, et al. 1995, Skott, et al. 2002, Turpin, et al. 1996). SLPI inhibits the HIV-1 infection after the virus binding, but not before reverse transcription (McNeely, et al. 1997).

Essentially, SLPI confers local protection against the microbial, fungal and HIV-1 insults. It is remarkable that only SLPI among all of the other proteins in saliva was found to be the most potent anti-HIV-1 factor. This fact provides an explanation for the scantiness and rarity of HIV-1 transmission via oral route. In addition, SLPI provides the limited tissue damage arisen due to inflammation, so it has a healing influence on wounds, as a precipitating agent. SLPI might be used as an indicator for the progress of infection or for the malignant lesions (Doumas, et al. 2005).

1.7. Aim of the Study

The aims of study are examining the status of SLPI protein production in the cells of human and other African Green Monkey (AGM) cells in the presence of HIV-1 tat protein, researching the effect of SLPI, on HIV-1 LTR promoter, which we have observed to increase in the presence of HIV-1 tat protein as a result of the studies we made on cv-1 cells of AGM which is known to be HIV-1 infection resistant, examining the status of SLPI protein production in the cells of humans and monkeys, infected by the HIV-1 molecular clone and examining the effect of SLPI protein on HIV-1 replication and researching if virus has anti-HIV effect after entering into the cell.

There are very limited works investigating the individual effects of HIV-1 tat and rev genes on mammalian cells. The biggest reason for this is that most of the HIV-1 molecular works are based on viral transduction works and viral vectors are used in researches. Hence, non-viral mammalian expression vector has been utilized to observe the individual effects of these genes in our study. Changes due to other genes have been eliminated because the entire genome of the virus has not been transferred. Although there are publications related to extracellular (inactivation of the virus before entering the cell) anti-HIV-1 effects of SLPI, there are no studies on intracellular (after integration of the virus) anti-HIV-1 mechanism thereof. Information as to the intracellular anti-HIV-1 effect of protein will be obtained if our assumption in the project also works with infected cells. This feature of our study makes it different than other similar works. Most of the publications as to SLPI in the literature have been conducted on human cells while there are no studies carried out on monkey cells. Our study's being carried out on monkey cells makes our project unique. Furthermore, our finding as to induction of SLPI in AGM CV-1 cell lines in the presence of HIV-1 tat protein in the preliminary works we have carried out makes this study worth to be published even at this stage. Our project envisaging a detailed study on this subject has the nature of providing unique contribution to the literature in this regard. If we prove that SLPI protein has anti-HIV effect after the virus enters into the cell, we believe that our project will provide significant improvements and contribution to health and vaccination fields which are priority areas and shed light on the cited fields.

If we can complete the project successfully it will be determined if production of SLPI protein in cell lines of humans and the old-world monkeys are related to HIV regulatory proteins or not and if there is a relation, the differences between cell lines resistant and sensitive against HIV-1 will be revealed and it will be shown if this difference has a role in the old-world monkeys' cells being resistant against HIV-1. We believe that revealing these differences/similarities will provide a major contribution scientifically. We aimed to reach data which will provide great benefit as to HIV -1 resistance mechanisms in monkey cells in addition to studies on only SLPI's highlighted extracellular anti-HIV-1 effect in literature, in case of finding a possible intracellular anti-HIV-1 effect of SLPI. Further, this effect will shed light to vaccination works carried out against HIV-1. We anticipate that the articles to be prepared in the light of the data to be obtained will be published in important domestic and international magazines published on this issue and we aim to share the relevant results in national and international congresses. We believe that our project has also importance in terms of creation of articles and papers in our country where there is little publications and work especially on molecular biology of HIV-1.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

A detailed list of commonly used chemicals, buffers, solutions and their compositions are presented in Appendix A.

2.1.1. Commercial Kits

Table 2.1. Commercial Kits.

Kits	Supplier Company
Tag DNA Polymerase	Fermentas
GeneJet RNA Purification Kit	Fermentas
RevertAid Firs Strand cDNA Synthesis Kit	Fermentas
InsTAclone PCR Cloning Kit	Fermentas
Clonejet PCR Cloning Kit	Fermentas
Dnase I Rnase-Free	Fermentas
Maxima Syber Green/Rox qPCR Master Mix 2X	Fermentas
TurboFect™ in vitro Transfection Reagent	Fermentas
MiniElute Gel Extraction Kit	Qiagen
High Pure Plasmid Extraction Kit Mini Prep	Roche
PureLink™ Plasmid DNA Purification Kits	Invitrogen
Nano-Glo Luciferase Assay System	Promega
Bradford Reagent	Fermentas
Mammalian Cell Lysis Buffer	Fermentas

2.1.2. Plasmids

We used six different plasmids in this study. These plasmids can be listed as pTZ57R/T, pJET1.2/blunt (Fermentas), pBudCE4.1 (Invitrogen), pBudCE4.1-Tat (Burcu Sengez Thesis), pNL3.2.NF-kappaB-RE (Promega) and pHIVlacZ (NIH AIDS) shown in the Figure 2.1-2.2-2.3-2.4-2.5-2.6.

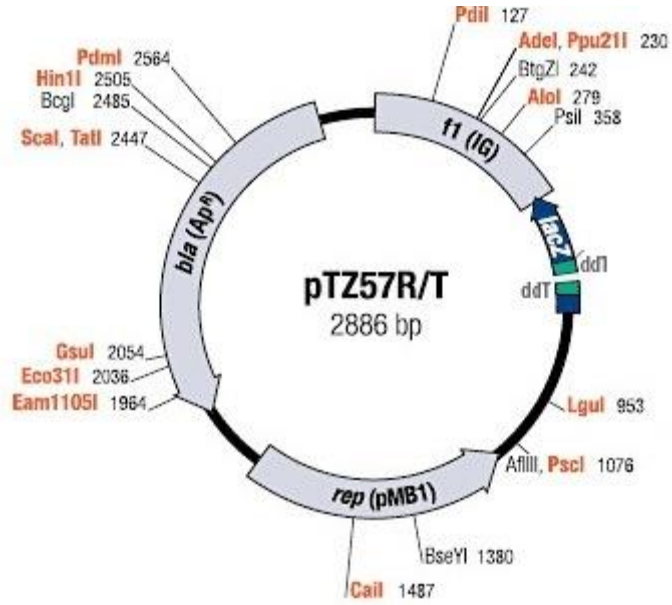

Figure 2.1. pTZ57R/T Sub-Cloning Plasmid (Fermentas).

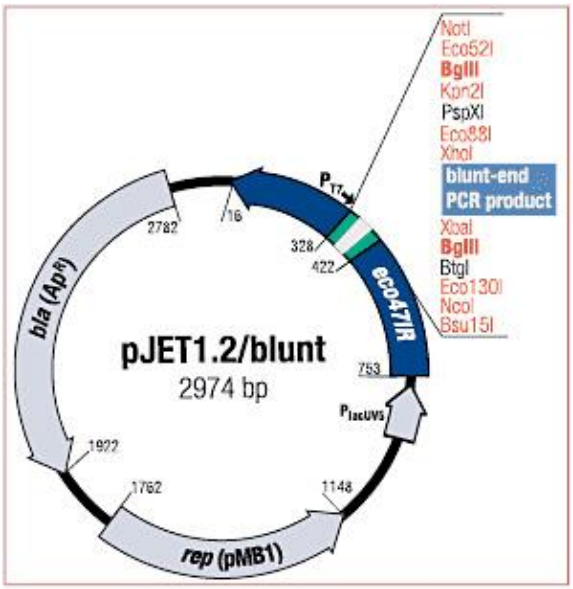

Figure 2.2. pJET1.2/blunt Sub-Cloning Plasmid (Fermentas).

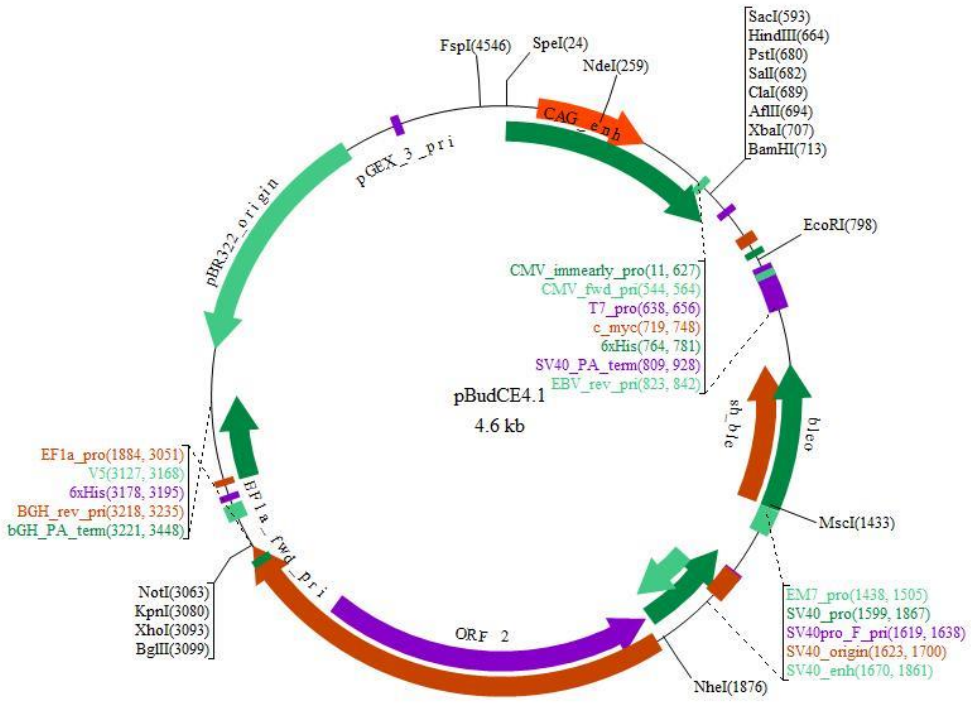

Figure 2.3. pBudCE4.1 Mammalian Expression Vector (Invitrogen).

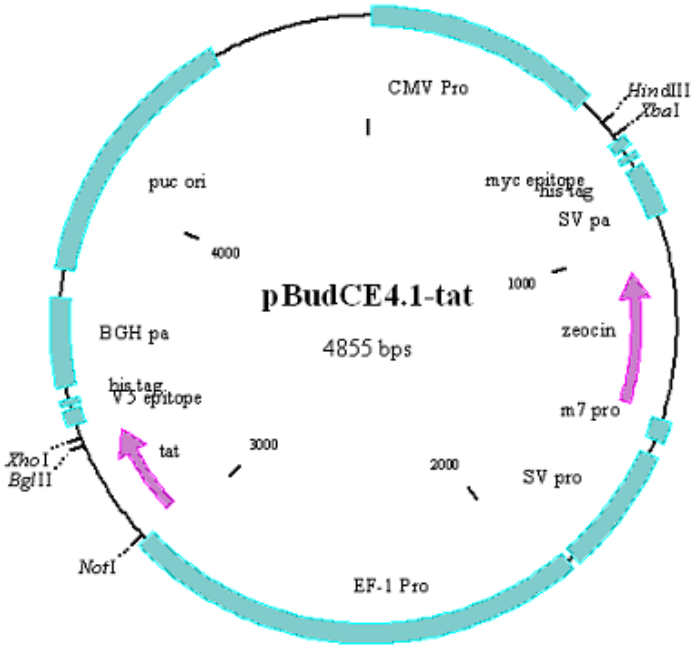

Figure 2.4. pBudCE4.1-tat Mammalian Expression Vector.
(Source: Burcu Sengez Thesis 2011)

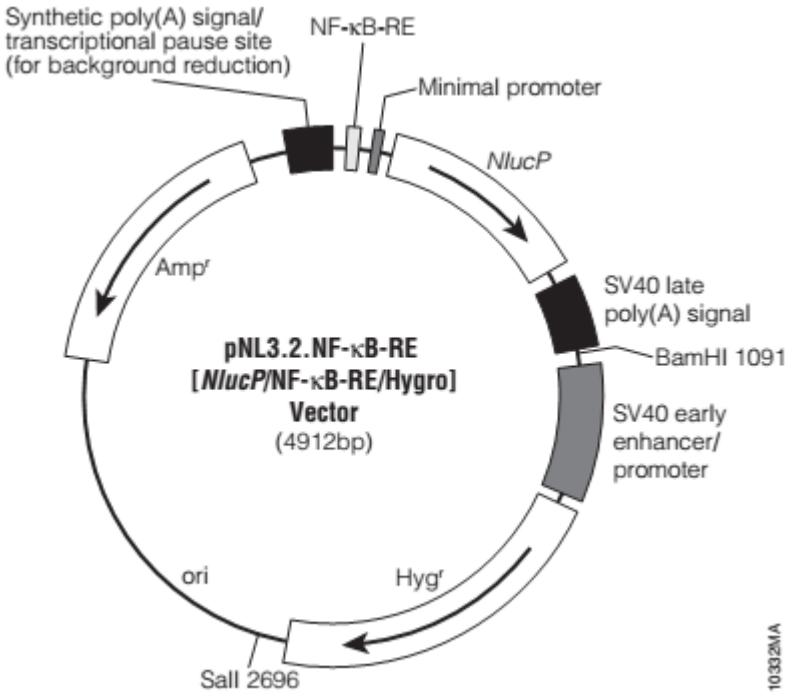

Figure 2.5. pNL3.2.NF-kB-RE [Nluc/NF-kB-RE/Hydro] Plasmid (Promega).

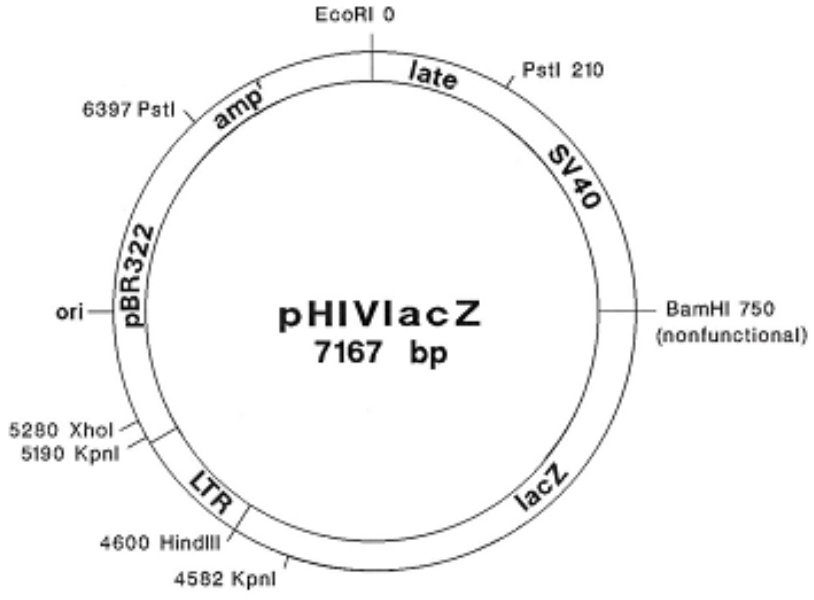

Figure 2.6. pHIVlacZ Plasmid (NIH AIDS).

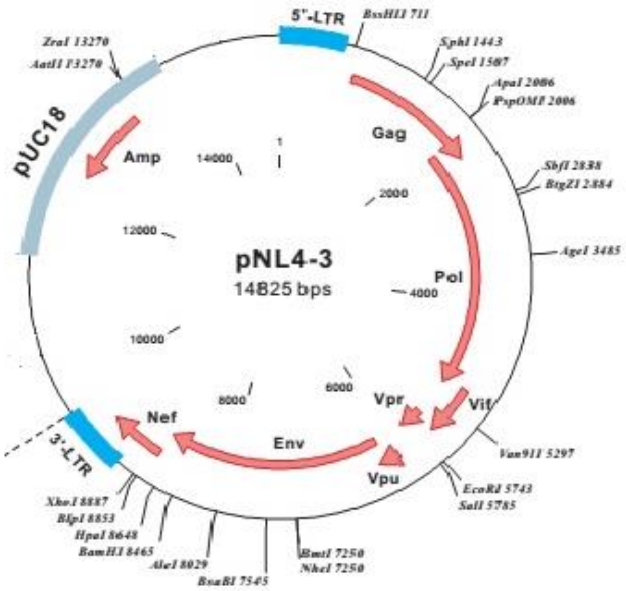

Figure 2.7. pNL4.3 HIV Plasmid (NIH AIDS).

2.1.3. Primers

We designed primers, which were ordered from Sentromer DNA Technology. The sequences of the primers used in this thesis study and their cleavage sites are given in Table 2.1.1. Real time PCR primers were designed via IDT SciTools and shown in the Table 2.2.

Table 2.2. Primer Sequences Used for Q RT-PCR

Primer Name	Sequence
PstI-SLPI-F	5'-CTGCAGCACACCATGAAGTCCAG-3'
XbaI-SLPI-R	5'-TCTAGATCAAGCTTTCACAGG-3'
EcoRI-Luc-F	5'-AAGCTTGCCACCATGGGTTTC-3'
HindIII-Luc-R	5'-GAATTCCCCAATACGGCAAACG-3'
qSLPI-F	5'-CTAGGGAAGAAGAGATGTTG-3'
qSLPI-R	5'-CCTAAATCACAGGAATC-3'
q-GAPDH-F	5'-TGCACCACCACCTGCTTAGC-3'
q-GAPDH-R	5'-GGCATGGACTGTGGTCATGAC-3'
q-ZEOCIN-F	5'-TGATGAACAGGGTCACGTCGT-3'
q-ZEOCIN-R	5'-AAGTTGACCAGTGCCGTTCCG-3'
HIV-LTR-F	5'-CCTTCTCGTCTAGATGGAAGGGCTAAA….CCC-3'
HIV-LTR-R	3'-CCTTCTCGGCTACCCTCCTTCTAGCC….TAG-5'

2.2. Cell Culture

CV1, COS7, Vero, Pindak, HeLa and 293 (HEK) cell lines were used in this study. The CV1 cell line is originated from the kidney cells of a *Cercopithecus aethiops*, which belongs to the African Green Monkeys (AGM). The COS7 cell line is originated from the same origin as the CV1 cells, but their only difference from the cv1 is that COS7 contains the genetic material of SV40. The Vero cell line is originated from the kidney epithelial cells of a *Chlorocebus sp*, which belongs to the African Green Monkeys (AGM). Pindak cell line is Squirall monkey fibroblast cell lines. The HeLa cell line is originated from the human cervical cancer cells. The 293 (HEK) cell line is originated from the human embryonic kidney cells.

2.2.1. Freezing & Thawing of Cells

First of all, the cell lines were taken out of the -80 ^{o}C and kept in a water-bath at 37 ^{o}C until being dissolved. Thereafter, these cells were added into a DMEM medium, which containing 10% FBS and 1% penicillin and located in a plate of 60 mm and then, put in an incubator at 37 ^{o}C and adjusted to 5% CO_2 and kept waited in this incubator for one day. One day later, medium of the cells was changed, in order to remove the DMSO. The cells were cared through passaging. New stocks were obtained from these cell lines. During the stocking process, the cells were firstly cultivated in the plates of 100 mm. The medium was removed at the time, when the plates were covered by the cells at rate of 80% and the cells were washed with 1X PBS and the cells were harvested by adding trypsin. Thereafter, the cells were centrifuged at 1500xg for 2 minutes and the cell pellet was dissolved with a 10% DMSO and 20% FBS. The dissolved cells were transferred into 1 mL cryogenic tubes. Thereafter, these tubes were firstly kept waited at -20 ^{o}C for one day and then, transferred into the liquid nitrogen at -196 ^{o}C, so that the cells can be stored for a long period.

2.2.2. Maintenance of Cells

CV1, COS7, Vero and Pindak cells were used in this study, were maintained with a DMEM, which containing 10% FBS and 1% penicillin streptomycin and a low degree of glucose and keeping in an incubator at 37 ^{o}C and adjusted to 5% CO_2. The HeLa and 293 (HEK) cells were maintained with a RPMI, which containing 10% FBS and 1% penicillin streptomycin and a high degree of glucose and keeping in an incubator at 37 ^{o}C and adjusted to 5% CO_2.

2.3. Transfection

The pBubCE4.1 (Invitrogen) and pBudCE41._*tat* (Burcu Sengez Thesis 2011) that we have created in our previous transfected to the CV1, Vero, COS7, Pindak, HeLa and 293 (HEK) cell lines using TurboFect™ in vitro Transfection Reagent (Fermentas)in proper way, as recommended by the manufacturer. One million cells for each cell lines have been used in the transfection process. In addition, plasmid of 1 microgram was transfected to each cell lines. After completion of the transfection processes, these cell have been kept waited for 48 hours. The level of transfection was measured with the RT-PCR method, using the Zeocin q PCR primers, as the pBudCE4.1 vector contains the Zeocin resistance gene.

2.4. Total RNA Isolation and cDNA Synthesis

For these experiments, CV1, Vero, COS7, Pindak, HeLa and 293 (HEK) cell lines were firstly transfected with HIV-1 *tat* gene, which is located in pBudCE4.1_*tat*. After 48 hours, HIV-1 tat gene transfected cell lines were harvested by adding trypsin. These cells were centrifuged at 1500xg for 2 minutes and the cells were precipitated. Thereafter, the total RNA was isolated from these cells with GeneJet RNA Purification Kit (Fermentas) in accordance with the procedure of this kit. After completion of the total RNA isolation process, the RNA concentrations were measured with the nanodrop. Thereafter, 1 microgram of the total RNA was treated with DNaseI (Fermentas), in order to exclude any DNA contamination. This process was applied in accordance with the procedure of the kit. 1 microgram of the total RNA treated with the

DNaseI was converted to the cDNA, using the RevertAid Firs Strand cDNA Synthesis Kit (Fermentas) and in accordance with the procedure of the kit. Oligo dt was used during this process. Thereafter, the cDNA amounts were measured with nanodrop and these cDNAs were diluted with nuclease-free water at rate of 1:10 and aliquot in a way to be 200 Nano-gram/microliter. Thereafter, the obtained cDNAs have been stored at -80 ^{o}C, to be used later. The same processes were applied to the Vero, COS7, CV1, Pindak, HeLa and 293 (HEK) cells, which do not contain any HIV-1 *tat* genes, in order to be used for control purposes.

2.5. Quantitative Real Time Polymerase Chain Reaction (Q RT-PCR)

The level of SLPI expression in the CV1, COS7, Vero, Pindak, HeLa and 293 (HEK) cells, which contain HIV-1 *tat* gene was measured with QRT-PCR. For this experiment, we designed primers, which amplified a part (245 bp) of the coding sequence of the SLPI gene. These primer sequences were shown in Table 2.2. At the same time and for the control purpose, the expression level of the Zeocin resistance gene located at the mammalian expression vector pbudCE4.1 was measured in order to determine the transfection efficiency, also GAPDH, which is known as house keeping gene expression level was measured with QRT-PCR for internal control. For these genes, we also designed the GAPDH and Zeocin primers. Each one of the primers were aliquoted in a way to be 5 micromolar/microliter. Initially, a master mix for the q-PCR was prepared in a nuclease-free Eppendorf of 1.5 microliter for each one of the genes. Master mix was contained 12.5 microliter Maxima Syber Green/Rox qPCR Master Mix 2X (Fermentas) and 1.5 microliter from the primer stocks of 5 uM/ul and 9 microliter nuclease free water. As a template, 2 microliters from each one of the cDNA of 200 ng/ul were used for each cell lines. Thereafter, the expression levels of these genes have been measured, using the real-time devices of Roche, which are available in the Izmir High Technology Institute and in the Biotechnological and Bioengineering Applications and Researches Center. This experiment has been repeated six times. PCR conditions used in the experiment were shown in Table 2.3.

Table 2.3. Q RT-PCR Conditions.

Steps	Temperature (^{0}C)	Duration (min:sec)	Cycle
Initial Denaturation	95	10:00	**1**
Denaturation	94	00:10	
Annealing	58	00:20	**40 cycles**
Extention	72	00:72	
Melting Curve	60	1:00	**1**

2.6. Analysis of Proteins

2.6.1. Protein Isolation

For the protein isolation, firstly, CV1,Vero, COS7, Pindak, HeLa and 293 (HEK) cell lines, which were transfected with HIV-1 *tat* gene when reached a sufficient degree of confluence. After 48 hours, HIV-1 *tat* gene transfected cell lines were harvested by adding trypsin. These cells were centrifuged at 1500xg for 2 minutes and the cells were precipitated. Thereafter, EDTA free anti-protease cocktail tablet was added into the Mammalian Cell Lysis Buffer (Fermentas). Then proteins were isolated from the cells with using this cell lysis buffer and in accordance with its procedure. The isolated cells have been stored at -80 ^{o}C, to be used later.

2.6.2. Bradford Assay

We performed a Bradford Assay, in order to measure the protein concentrations. Firstly, BSA concentrations of 10, 20, 50, 100, 150 and 200 µg/ml were prepared for this assay. Thereafter, 40 ml from the each one of the prepared BSA was added and placed into a plate of 96 wells and 160 ml from the Bradford solution (Fermentas) was added onto them. And the measurements were taken at the wave length of 595 nm with the spectrophotometer. A standard curve was created in the excel format in accordance to the results of these measurements. Concentrations of the isolated proteins were determined, using this standard curve, which was shown in Figure 2.8.

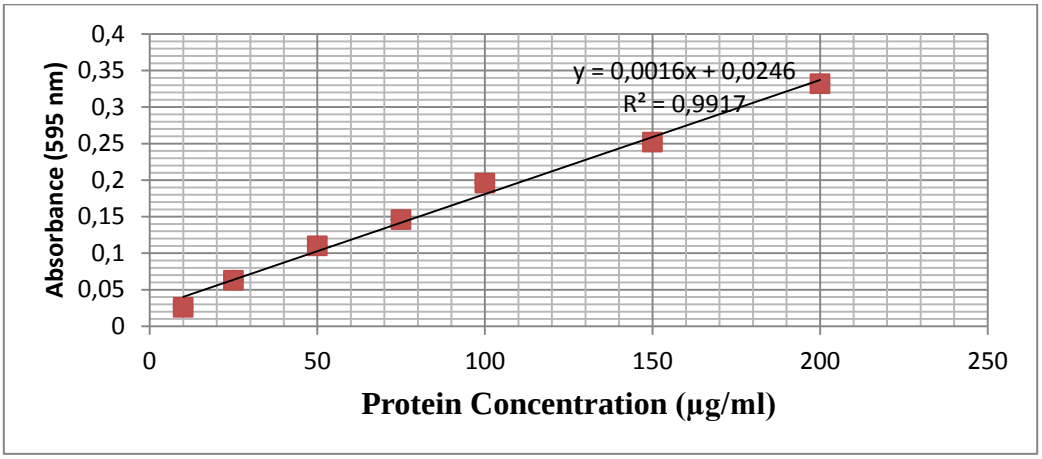

Figure 2.8. BSA Standard Curve Graphic

2.6.3. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

First we optimized western blot protocol in our laboratory then we detected our SLPI proteins using western blot technique. We prepared SDS-PAGE gel to run and separate our proteins accordance to molecular weight. We prepared 4% upper gel and 12% resolving gel (dH_20, upper buffer or resolving buffer, %30 Acrylamide (Sigma), 10% SDS (Applichem), TEMED (Sigma) , 10% APS (Applichem)). 25 µg protein mixture was mixed with protein loading dye and heated at 90 °C at 10 minutes. Protein mixture was loaded on gel and run 2 hours at 100V as vertical electrophoresis (BioRad) in running buffer (0.25M Tris, 1,92 M Glycine, 1% SDS (w/v)).

Another gel was prepared to stain proteins with 1 mg/ml Coomasie Blue (Sigma), 10% acetic acid, 30% methanol or ethanol. Gel was incubated in Coomasie Blue for 1 hour and incubated in the same solution without Coomasie Blue to visualize protein bands in order to confirm whether lysis method was worked or not.

After electrophoresis, gel was placed between whatmann sheet and nitrocellulose membrane. Nitrocellulose membrane was activated in transfer buffer (48mM Tris, 39mM Glycine, 20% ethanol (v/v), 1L dH_2O).

Proteins were transfered to nitrocellulose membrane (BioRad) at 60 V for 3 hours. Membrane washed ultrapure water and washing buffer (1X PBS, 0.05% Tween20).

Membrane was blocked with blocking buffer (1X PBS, 0.05% Tween20, 0,5% non-fat dry milk) for 1 hour at room temperature. After blocking, membrane was incubated with washing buffer including 10% blocking buffer with anti-SLPI (1/1500) (Pierce) and GAPDH primer antibodies (1:5000) (Pierce) in different containers for 1 hour and shaking gently. Membranes were washed 3 times with washing buffer for 5 minutes each and membrane was incubated in washing buffer including 10% blocking buffer with anti-rabbit HRP (Horseradish peroxide) seconder antibody (Pierce 1:5000) for 1 hour and shaking gently. Membranes were washed 6 times for 5 minutes each. After last washing, 300 µl enhanced chemiluminescent substrate for HRP (Thermo) and 300µl enhancer solution (Thermo) were spread on membrane and incubated for 3 minutes. Membranes releasing chemiluminescence were visualized using VersaDoc Imager in Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center.

2.7. Total RNA isolation, cDNA synthesis and PCR Amplification of Fragment of SLPI Gene

Total RNA were isolated from cos7 AGM cells with GeneJet RNA Purification Kit (Fermentas) according to manufacturer's instructions. This RNA was treated to DnaseI Rnase Free (Fermentas) to eliminate DNA contamination. Following cDNA was synthesized from this RNA by using RevertAid Firs Strand cDNA Synthesis Kit (Fermentas) according to manufacturer's instructions. This cDNA was used in Polymerase Chain Reaction (PCR) with Tag DNA Polymerase (Fermentas). SLPI fragment was amplified with specific primers designed before in the mixtures and conditions as shown in Table 2.4 and Table 2.5, respectively.

Table 2.4. The Amount of PCR Components Respectively Reaction Mix.

Solutions	PCR mix µl
dH_2O	16.2 µl
10X Buffer	2.5 µl
$MgCl_2$	2 µl
dNTPs	2 µl
SLPI primer F	1 µl
SLPI primer R	1 µl
cDNA	2 µl
Taq Polymerase	0.3 µl

Table 2.5. PCR Conditions.

Cycle Number	Cycle	Temperature (^{0}C)	Duration
1	Initial Denaturation	95	2 minutes
	Denaturation	94	30 seconds
30	Annealing	58	30 seconds
	Extention	72	1 minutes
1	Final Extention	72	5 minutes

After SLPI gene fragments amplified with PCR, it was run in 1% agarose gel. Fragment was purified using MiniElute Gel Extraction Kit (Qiagen) from 1% agarose gel.

2.7.1. TA Cloning and Confirmation with Restriction Enzyme Digestion

Purified fragment was cloned with InsTAclone PCR Cloning Kit (Fermentas) to pTZ57R/T subcloning vector according to manufacturer's instructions. MAX Efficiency® DH5α-T1 Chemically Competent *E. coli* cells (Invitrogen, 12297-016)

were used for bacterial transformation. White colonies were selected by blue-white screening. These selected colonies were inoculated into LB-broth medium containing 100 µg/ml ampicilin and incubated at 37 ^{0}C in a shaker incubator for overnight. Plasmids were purified using High Pure Plasmid Extraction Kit Mini Prep (Roche). Purified plasmid were digested by PstI and XbaI restriction enzyme at 37 ^{0}C for 1 hours for conformation of TA cloning as shown in Table 2.6. Digestion mixture was run on 1% agarose gel for detection of confirmation.

Table 2.6. The Amount of Double Digestion Components Respectively Reaction Mix.

Solutions	Reaction Mix
10X Buffer	2 µl
Vector	5 µl
PstI	2 µl
XbaI	1 µl
dH_2O	10 µl

2.7.2. Cloning of SLPI Fragment to pBudCE4.1 Mammalian Expression Vector

Firstly, we double digested pBudCE4.1 (Invitrogen) with PstI and XbaI at 37 ^{0}C for 1 hours. At the end of the incubation, digestion mixture was run on 1% agarose gel to observe digestion. Following, this vector was purified with MiniElute Gel Extraction Kit (Qiagen) from 1% agarose gel according to manufacturer's instructions. Digested pBudCE4.1 was used for cloning of SLPI fragments. pTZ57R/T vector including our insert was digested with PstI and XbaI at 37 ^{0}C for 1 hours. At the end of digestion, our insert was run on 1% agarose gel and was purified with MiniElute Gel Extraction Kit (Qiagen) from 1 % agarose gel according to manufacturer's instructions and its concentration was measured with nanodrop. Purified insert was used in ligation with double digested pBudCE4.1 with T4 Ligase (Fermentas) according to manufacturer's instructions as shown Table 2.7.

Table 2.7. The Amount of Ligation Components Respectively Reaction Mix.

Solutions	Ligation Mix
Buffer T4 DNA ligase (10X)	2 µl
pBudCE4.1 expression vector	5 µl
Extracted SLPI gene from gel	12 µl
T4 DNA ligase	1 µl

After ligation, 1 microliters of ligation mixture was transformed into MAX Efficiency® DH5α-T1 Chemically Competent *E. coli* cells (Invitrogen, 12297-016) according to manufacturer's instructions and bacterial cells were plated on 100 µg/ml Ampcillin LB-agar plates following 16 hours incubation at 37 ^{0}C. Transformation produced colonies on the plates and they were inoculated into 100 µg/ml Ampcillin LB-broth medium for 16 hours at 37 ^{0}C. After overnight incubation, cloned pBudCE4.1 vectors were isolated from bacterial cultures with High Pure Plasmid Extraction Kit Mini Prep (Roche) according to manufacturer's instructions. These plasmids were confirmed by double digestion with PstI and XbaI at 37 ^{0}C for 1 hours.

Cloned pBudCE4.1 plasmids containing SLPI gene fragment was sequenced at Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center as a service provider by different fluorescent dye labelled dideoxynucleotide chainterminating method. Cloned pBudCE4.1 vectors were sequenced with T7 primer.

2.8. Cloning of NanoLuciferase Gene to pHIVlacZ Vector

The gene of NanoLuc (820 bp) is located within the pNL3.2.NF-kB-RE[Nluc/NF-kB-RE/Hydro] vector (Promega). For this reason, this vector was used in Polymerase Chain Reaction (PCR) with Tag Polymerase (Fermentas). NanoLuc gene was amplified with primers designed before (Table 2.2) in the mixture and conditions as shown Table 2.8 and Table 2.9, respectively.

Table 2.8. The Amount of PCR Components Respectively Reaction Mix.

Solutions	PCR mix µl
dH_2O	16 µl
10X Buffer	2.5 µl
$MgCl_2$	2 µl
dNTPs	2 µl
Luciferase-F	1 µl
Luciferase-R	1 µl
pNL3.2.NF-kB-RE [Nluc/NF-kB-RE/Hydro]	2 µl
Taq Polymerase	0.5 µl

Table 2.9. PCR Conditions.

Cycle Number	Cycle	Temperature (^{0}C)	Duration
1	Initial Denaturation	95	3 minutes
	Denaturation	94	30 seconds
30	Annealing	55	30 seconds
	Extention	72	1 minutes
1	Final Extention	72	5 minutes

After NanoLuc gene amplified with PCR, it was run on 1% agarose gel. Following, it was purified using MiniElute Gel Extraction Kit (Qiagen) from 1% agarose gel.

Purified Nanoluc gene was clone to pJET1.2/blunt vector (Fermentas) with Clonejet PCR Cloning Kit (Fermentas) according to manufacturer's instructions. pHIVlacZ (NIH AIDS) vector was double digested with EcoRI and HindIII at 37 ^{0}C for 1 hours. At the end of the incubation, digested pHIVlacZ was run on 1% agarose gel. Following, 2567 bp fragment which includes HIV-LTR region was purified with MiniElute Gel Extraction Kit (Qiagen) from 1% agarose gel. pJET1.2/blunt vector including Nanoluc gene was digested with MunI and HindIII restriction enzyme at 37

^{0}C for 1 hours. After incubation, digested Nanoluc gene was run on 1% agarose gel. Following, Nanoluc gene was purified with MiniElute Gel Extraction Kit (Qiagen) from 1% agarose gel. Purified Nanoluc gene was used in ligation with 2567 bp fragment of pHIVlacZ with T4 DNA Ligase (Fermentas) according to manufacturer's instructions as shown Table 2.10.

Table 2.10. The Amount of Ligation Components Respectively Reaction Mix.

Solutions	Ligation Mix
Buffer T4 DNA ligase (10X)	2 µl
2567 bp fragment of pHIVlacZ	7 µl
Extracted Nanoluc gene from gel	10 µl
T4 DNA ligase	1 µl

Following the ligation reaction, 2.5 µl ligation mixture was used in transformation to *E. coli* DH5α strain and bacterial cells bacterial cells were plated on 100 µg/ml Ampcillin LB-agar plates following 16 hours incubation at 37 ^{0}C. Transformation produced colonies on the plates and they were inoculated into 100 µg/ml Ampcillin LB-broth medium for 16 hours at 37 ^{0}C. After overnight incubation, constructed vector (pHIVluc) were isolated from bacterial cultures with High Pure Plasmid Extraction Kit Mini Prep (Roche) according to manufacturer's instructions. These plasmids were confirmed by double digestion with Eco81I and PstI at 37 ^{0}C for 1 hours.

Constructed pHIVluc plasmid containing Nanoluc gene was sequenced at Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center as a service provider by different fluorescent dye labelled dideoxynucleotide chainterminating method. Constructed pHIVluc vectors were sequenced with 5' HIV-LTR and 3' HIV-LTR primer.

2.9. Luciferase Assay

Luciferase assay has been carried out, in order to research and inspect the effect of the SLPI on the NFkB promoter and the HIV-1 LTR. The empty pbudCE4.1, pbudCE4.1_tat, pbudCE4.1_SLPI and pNL3.2NFkB (Promega) plasmids have been used in this experiment. Firstly, to investigate effect of the SLPI on NFkB promoter, 293 (HEK) and CV1 cells have been cultivated in the equal numbers ($15x10^3$) into 96 well plates. In 96 well plate, were prepared as Blank which was cultivated 293 (HEK) and CV1 cells and growth medium (DMEM) without any transfection, wells were prepared as a negative control which includes 293 (HEK) and CV1 cells transfected the empty vector pbudCE4.1, twelve wells were prepared as test samples co-transfected pNL-NFkB and pbudCE4.1_SLPI, pNL-NFkB and pbudCE4.1_tat plasmids into the 293 (HEK) and CV1 cell line. After 24 hours following the transfection, the medium has been removed and the cells have been induced by TNF alpha for 5 hours. Thereafter, medium has been removed and Nanoglo luciferase buffer and substrate mixture (Promega) has been added to cell lines and incubated 5 minutes. Then we measured luminance level for 10.000 mc by using varioscan. Secondly, to investigate effect of the SLPI on the HIV LTR, In 96 well plate, wells were prepared as Blank which has cultivated CV1, COS7, Vero, Pindak, Hela, 293 (HEK) cells and growth medium (DMEM) without any transfection, wells were prepared as test samples co-transfected pHIVluc and pbudCE4.1_SLPI, pHIV-luc and pbudCE4.1_tat plasmids into the all cell lines. After 24 hours following the transfection, the medium has been removed and the cells have been induced by TNF alpha for 5 hours. Thereafter we performed same process as describe above.

2.10. HIV-1 Infection Experiments

2.10.1. Real time PCR

SLPI, GAG and TAT gene expression levels were measured with real time PCR in the HIV-1 infected CV1 and 293 (HEK). In this experiment, CV1 and 293 (HEK) cells were transfected with pNL4.3 plasmid. After 48h, cells were harvested with adding

trypsin and total RNA and genomic DNA were performed from infected cells by using GeneJet RNA Isolation Kit (Fermentas) and Extraction Kit (Qiagen) according to manufacturer protocol respectively. We then carried out Real Time PCR for SLPI, GAG and TAT gene by using Qiagen Syber Green Master Mix according to manufacturer protocol.

2.10.2. ELISA p24 Antigen Test

We used INNOTEST® HIV Antigen mAb ELISA kit in this experiment, also CV1 and 293 (HEK) cells were used. HIV-1 molecular clone pNL4.3 plasmid (NIH AIDS) and pBudCE4.1_SLPI plasmid were transfected to these cells. Control group cells were only transfected with pNL4.3, other groups were transfected with both pNL4.3 and pBuedCE4.1_SLPI. After 48h, cell medium was collected and diluted at the rate of 1/1000, 1/10000 and 1/100000. Diluted cell mediums were added to the ELISA microtiter plate wells then conjugate 1 was added to the wells. After 1h, all wells were washed five times then conjugate 2 was added to the wells. After 30 minutes, stop solution was added to the wells. After incubation, p24 antigen concentration was measured with spectrometer at 450-680 nm. These experiments were carried out in BCL3 laboratory at Council of Turkish Public Health in ANKARA.

2.11. Statistical Analysis

Statistical significance was determined using one-way analysis of variance for quantitative QRT-PCR and Luciferase Assay. $P<0.05$ was considered to be significant.

CHAPTER 3

RESULTS

3.1. Determination of Zeocin Resistance Gene Expression

Zeocin resistance gene was located in pBudCE4.1 mammalian expression vector (Invitrogen). For this reason, zeocin mRNA expression in CV1, COS7, Vero, Pindak, HeLa and 293 (HEK) cells was examined by real-time QRT-PCR to observe transfection efficiency. Cv1, COS7, Vero, Pindak, HeLA and 293 (HEK) were transfected with PbudCE4.1_tat and pBudCE4.1 empty vector for 36 hours prior to RNA isolation, RT, and quantitative PCR amplification. Zeocin mRNA expression was increased in transfected cos7, vero and 293t cells. This result indicates that these cells were transfected with HIV-1 *tat* gene (Figure 3.1). Student's t-test was performed for compare the mRNA amount of each sample. Zeocin mRNA expression level was not statistically different from each other ($p>0.05$).

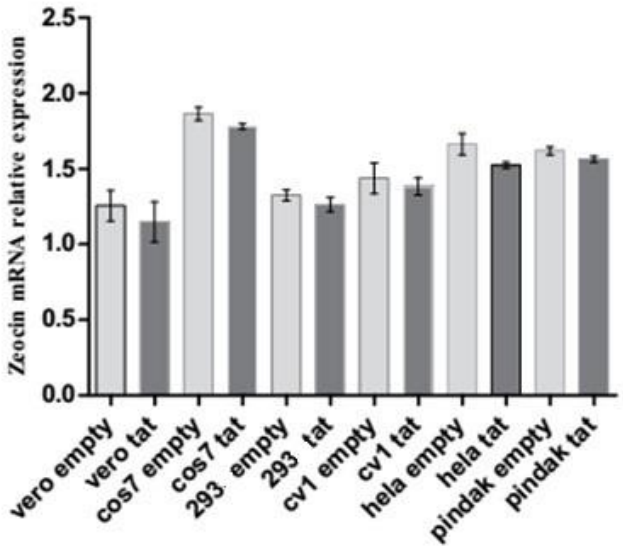

Figure 3.1. Determination of Zeocin mRNA expression by QRT-PCR. This chart shows the Zeocin mRNA expression level for each sample respectively by C. The relative expression level was calculated by using mean CT values of each sample ($p>0.05$).

3.2. Determination of SLPI Gene Expression

SLPI mRNA expression in AGM and human cells in response to HIV-1 tat gene was investigated by real-time QRT-PCR. Here, AGM and human cells were transfected with HIV-1 *tat* gene for 36 hours prior to RNA isolation, RT, and quantitative PCR amplification. Change in SLPI expression was measured by comparing the fold change in SLPI expression relative to GAPDH expression (internal control) for each lysate. Compared to mock transfected AGM cells, SLPI mRNA level rapidly increased in HIV-1 *tat* gene transfected CV1, COS7 and Vero cell lines. However, SLPI mRNA level was not increased in HIV-1 *tat* gene transfected human cells compare to mock transfected human cells. Student's t-test was performed for compare the mRNA amount of each sample. The expression level of SLPI mRNA in mock CV1, COS7 and Vero cell lines was significantly ($p<0.05$) lower than HIV-1 *tat* gene transfected CV1, COS7 and Vero cell lines. However, the expression level of SLPI mRNA in the mock HeLa, 293 (HEK) and Pindak cells was not statistically different from HIV-1 *tat* gene transfected Hela, 293 (HEK) and Pindak cell ($p>0.05$). These results indicate that HIV-1 *tat* gene induced SLPI expression in AGM cells but not induced in human cells. In addition, we analyzed QRT-PCR products with agarose gel electrophoresis to observe them. We detect SLPI, Zeocin and GAPDH genes on agarose gel.

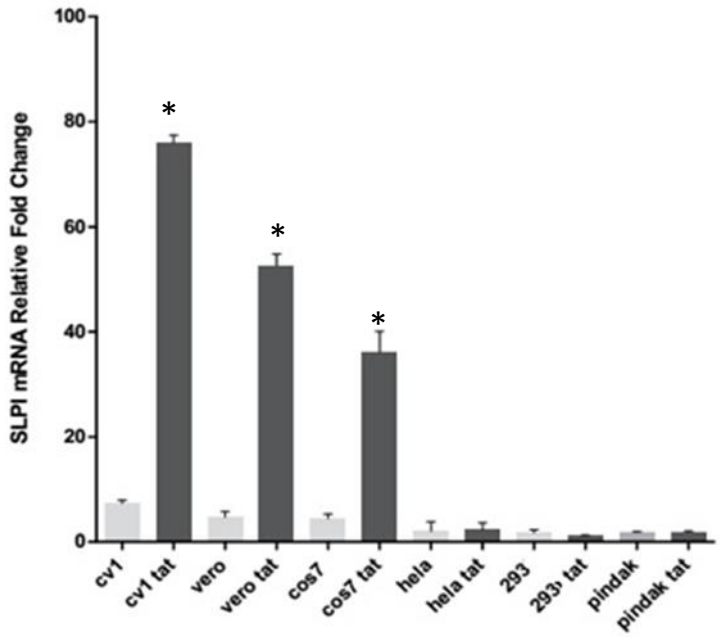

Figure 3.2.1. Determination of SLPI mRNA expression by QRT-PCR. This chart shows the SLPI mRNA expression level for each sample by two-step QRT-PCR. The relative SLPI expression level was calculated by using mean CT values of each sample (*:$p<0.05$).

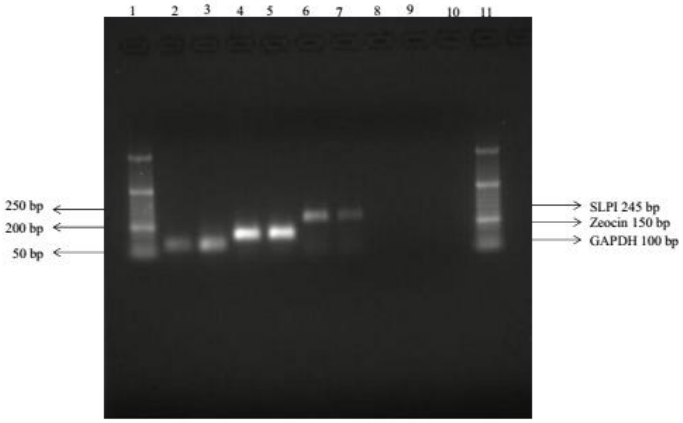

Figure 3.2.2. Agarose gel analysis of amplified QRT-PCR products. Line 1: 50 bp marker (Fermentas). Line 2: CV1 tat GAPDH. Line 3: CV1GAPDH. Line 4: CV1tat Zeocin. Line 5: CV1 Zeocin. Line 6: CV1 tat SLPI. Line 7: CV1SLPI. Line 8: Non-template control GAPDH. Line 9: Non-template control Zeocin. Line 10: Non-template control SLPI. Line 11: 50 bp marker (Fermentas).

3.3. Western Blot Analysis of SLPI Protein in AGM and Human Cells

We carried out Western Blot analysis to detect the SLPI protein in AGM and Human cells. AGM and human cells were transfected with HIV-1 *tat* gene for 36 hours prior to Western Blot analysis. SLPI protein was observed as 11.7 kDa particularly HIV-1 *tat* gene transfected AGM cells. Furthermore, we observed GAPDH protein as loading control. SLPI protein expression level was high in the HIV-1 *tat* transfected CV1, COS7 and Vero cells compare to empty pBudCE4.1 plasmid (Invitrogen) transfected CV1, COS7 and Vero. However, the expression level of SLPI protein was not changed in the HIV-1 *tat* gene transfected HeLa and 293 (HEK) cells compare to empty pBudCE4.1 plasmid (Invitrogen) transfected HeLa and 293 (HEK). These results indicate that SLPI protein expression increase in the HIV-1 *tat* gene transfected AGM cells and not increase in the HIV-1 *tat* gene transfected human cells. In addition, these results confirm our QRT-PCR results.

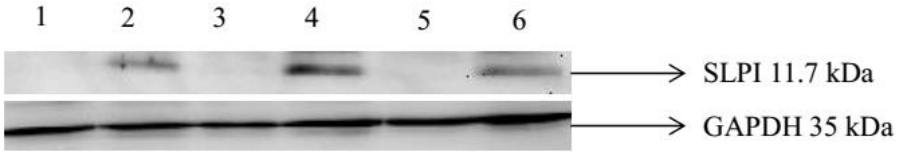

Figure 3.3.1. Western Blot Analysis of SLPI protein in AGM cells. Line 1: Empty COS7 cells. Line 2: HIV-1 tat gene transfected COS7cells. Line 3: Empty CV1 cells. Line 4: HIV-1 tat gene transfected CV1 c cells. Line 5: Empty Vero cells. Line 6: HIV-1 tat gene transfected Vero cells.

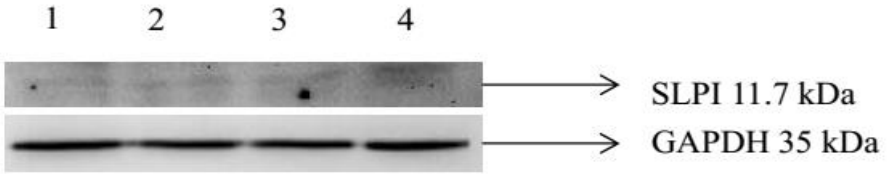

Figure 3.3.2. Western Blot Analysis of SLPI protein in human cells. Line 1: Empty HeLa cells. Line 2: HIV-1 *tat* gene transfected HeLa cells. Line 3: Empty 293 (HEK) cells. Line 4: HIV-1 *tat* gene transfected 293 (HEK) cells.

3.4. Construction of AGM SLPI ($SLPI_{agm}$) Expression Vector

In order to cloning of SLPI, initially we carried out PCR with specific primers, which involve PstI and XbaI restriction sites to amplify the $SLPI_{agm}$ cDNA sequence (Figure 3.4.1). Thereafter, $SLPI_{agm}$ cDNA sequence, which is amplified by PCR, was purified and introduced into the pTZ57RT (Fermentas) cloning vector as describe in methods part. This clone was called pTZ57RT/ $SLPI_{agm}$ (Figure 3.4.2). The insert of pTZ57RT/ $SLPI_{agm}$ was cleavage with PstI and XbaI restriction enzymes and cloned to pBudCE4.1 mammalian expression vector (Invitrogen) as describe in methods part. Our insert was also located into the CMV promoter. This clone was called pBudCE4.1 $SLPI_{agm}$ (Figure 3.4.3)

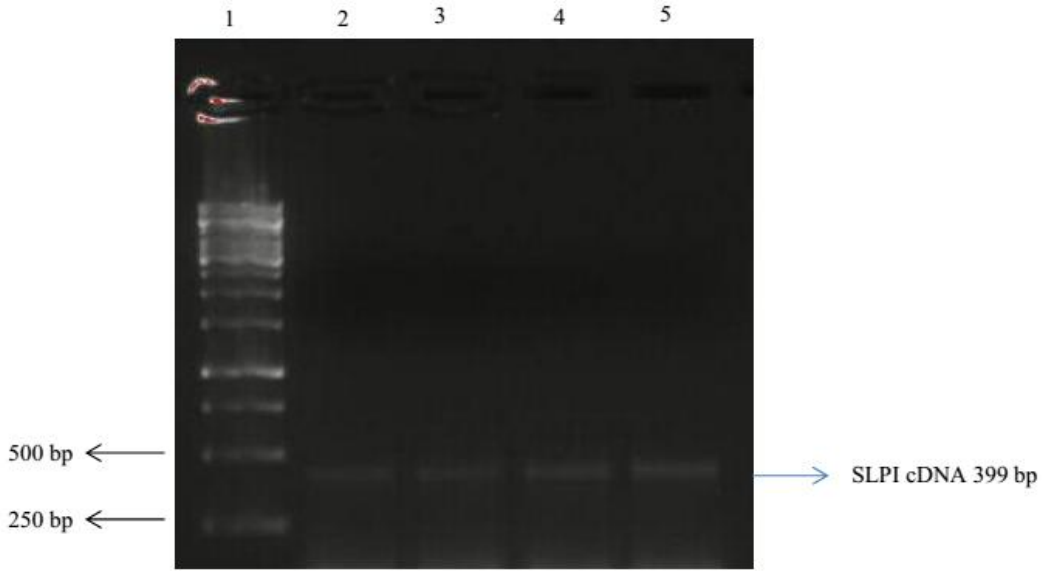

Figure 3.4.1**.** Agarose gel analysis of amplified $SLPI_{agm}$ cDNA sequence**.** Line 1: 1 kb DNA ladder (Fermentas). Line 2: CV1 cells cDNA template. Line 3: COS7 cells cDNA template. Line 4: Vero cells cDNA template. Line5: 293 (HEK) cells cDNA template.

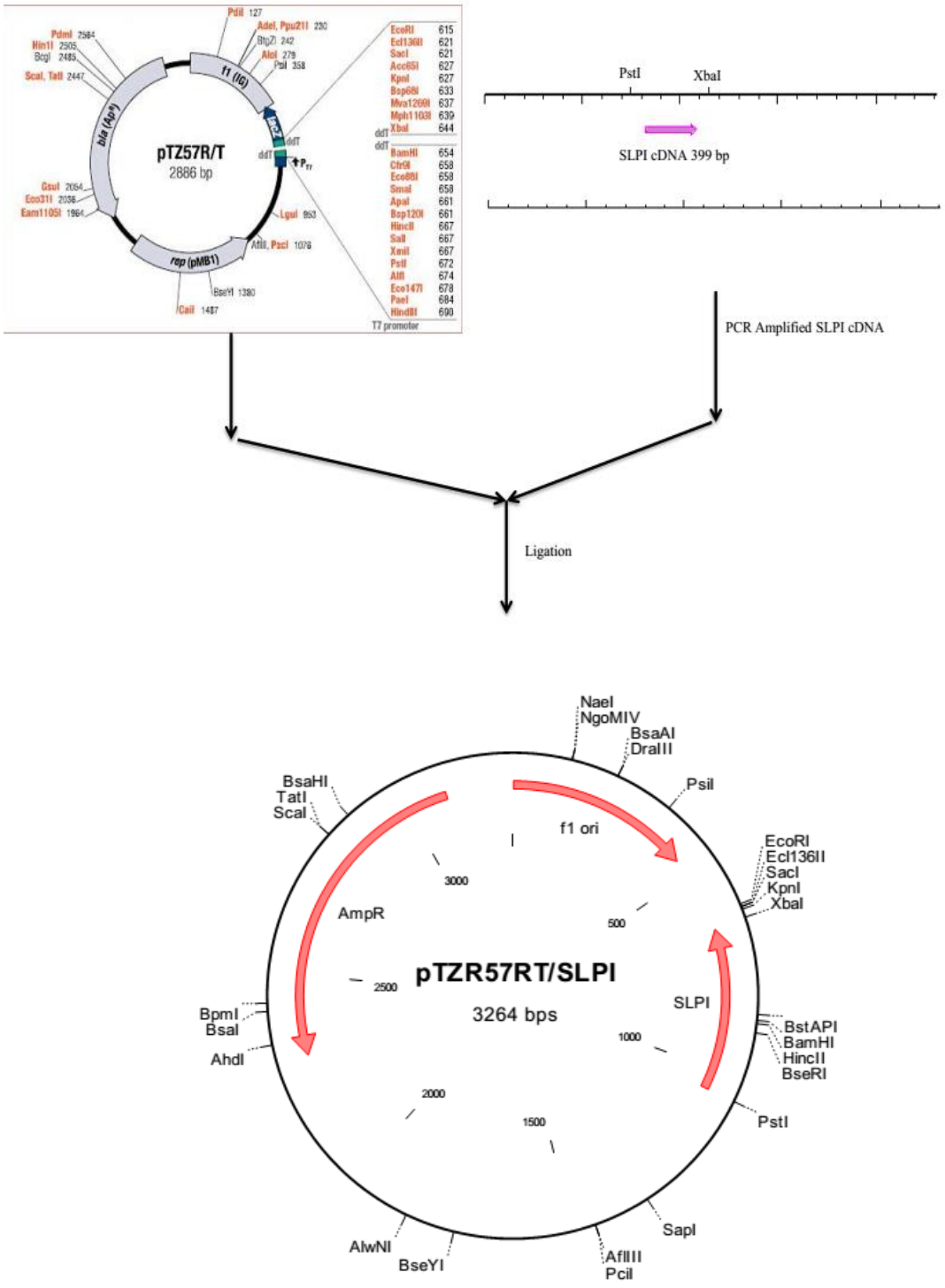

Figure 3.4.2. Construction of pTZ57RT/ $SLPI_{agm}$ Vector

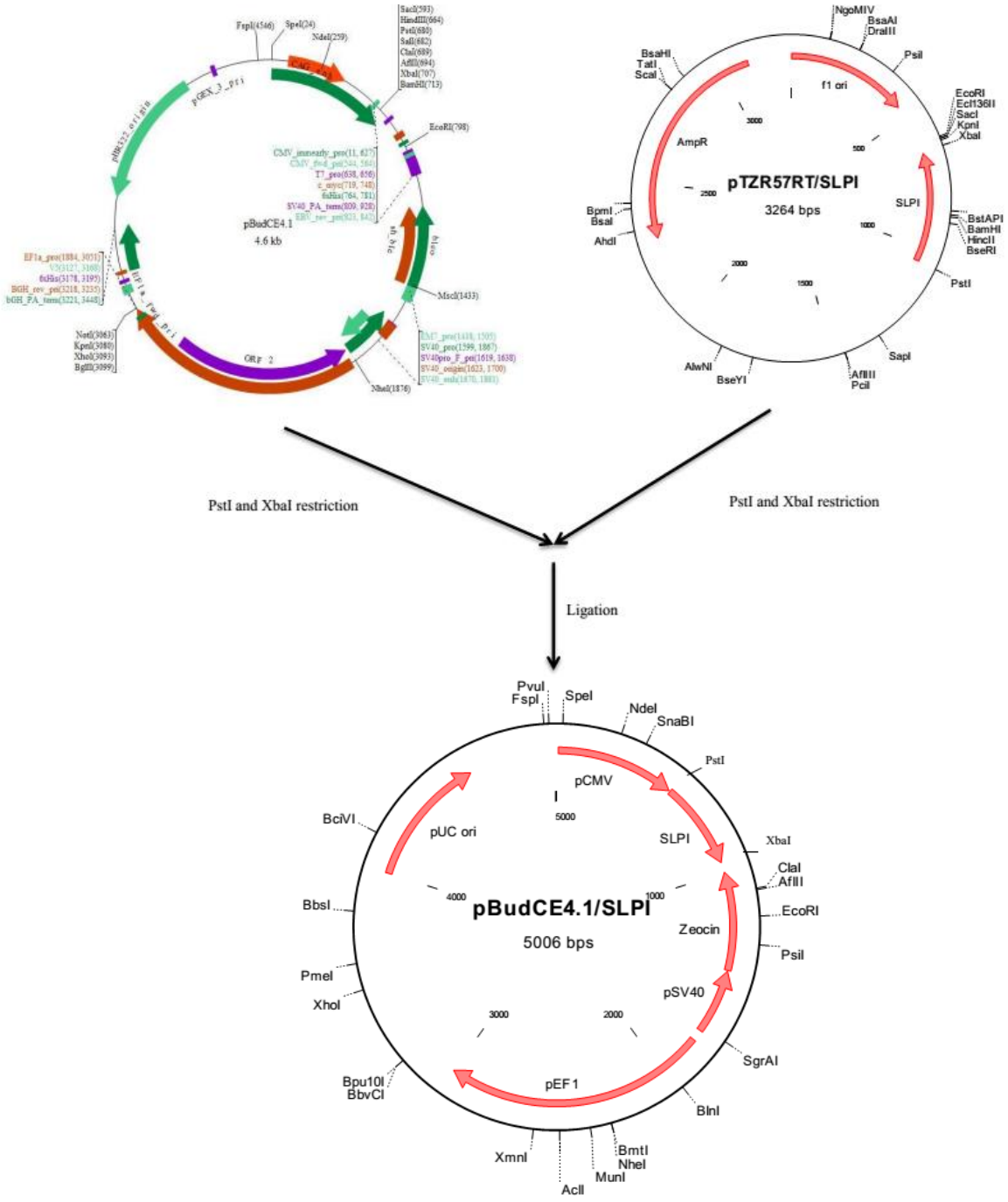

Figure 3.4.3. Construction of pBudCE4.1/ $SLPI_{agm}$ Vector

3.5. Construction of pHIVNluc Expression Vector

Initially nanoluciferase (Nluc) sequence was amplified with PCR using specific primers, which contain HindIII and EcoRI restriction enzymes. Since nanoluciferase gene is located into the pNL3.2.NF-kB-RE[Nluc/NF-kB-RE/Hydro] vector (Promega), we used this vector as a template in PCR reaction. Thereafter, amplified nanoluciferase

sequence was cloned to pJET1.2 cloning vector as describe in methods part. This clone was called pJET1.2/Nluc. The insert of the pJET1.2/Nluc, which was cleavage with HindIII and MunI restriction enzymes, was cloned to pHIVlacZ vector, which was cleavage with HindIII and EcoRI restriction enzymes as describe in methods part. This clone was called pHIVNluc expression vector.

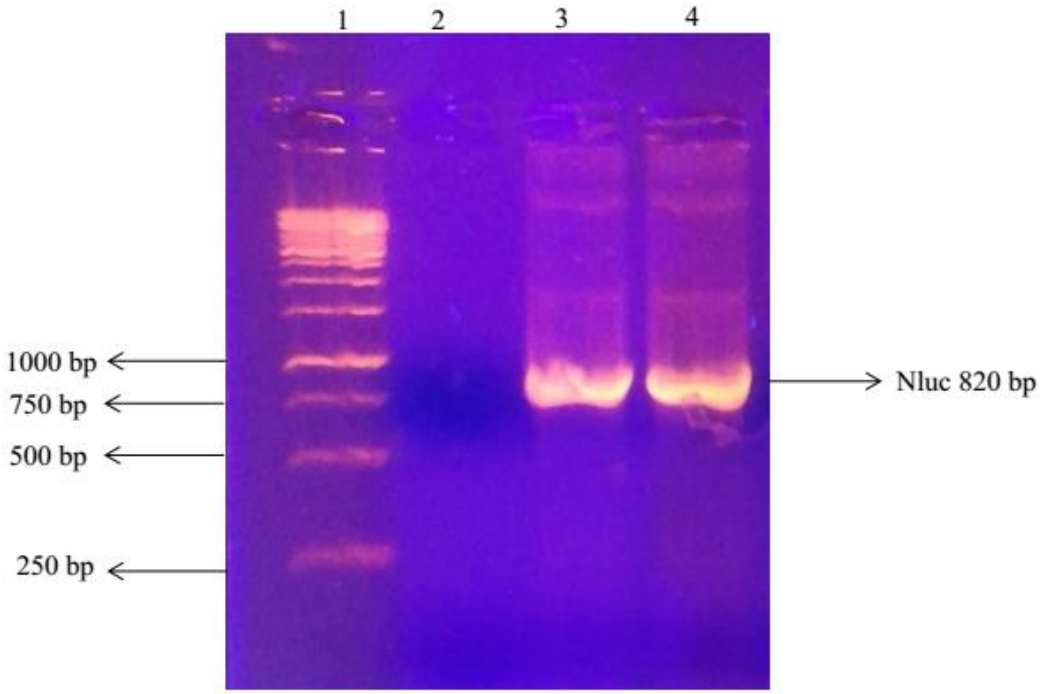

Figure 3.5.1. Agarose gel analysis of amplified Nluc sequence. Line1: 1 kp DNA ladder (Fermentas). Line 2: Non-template control. Line 3 and 4: pNL3.2.NF-kB-RE[Nluc/NF-kB-RE/Hydro] vector (Promega).

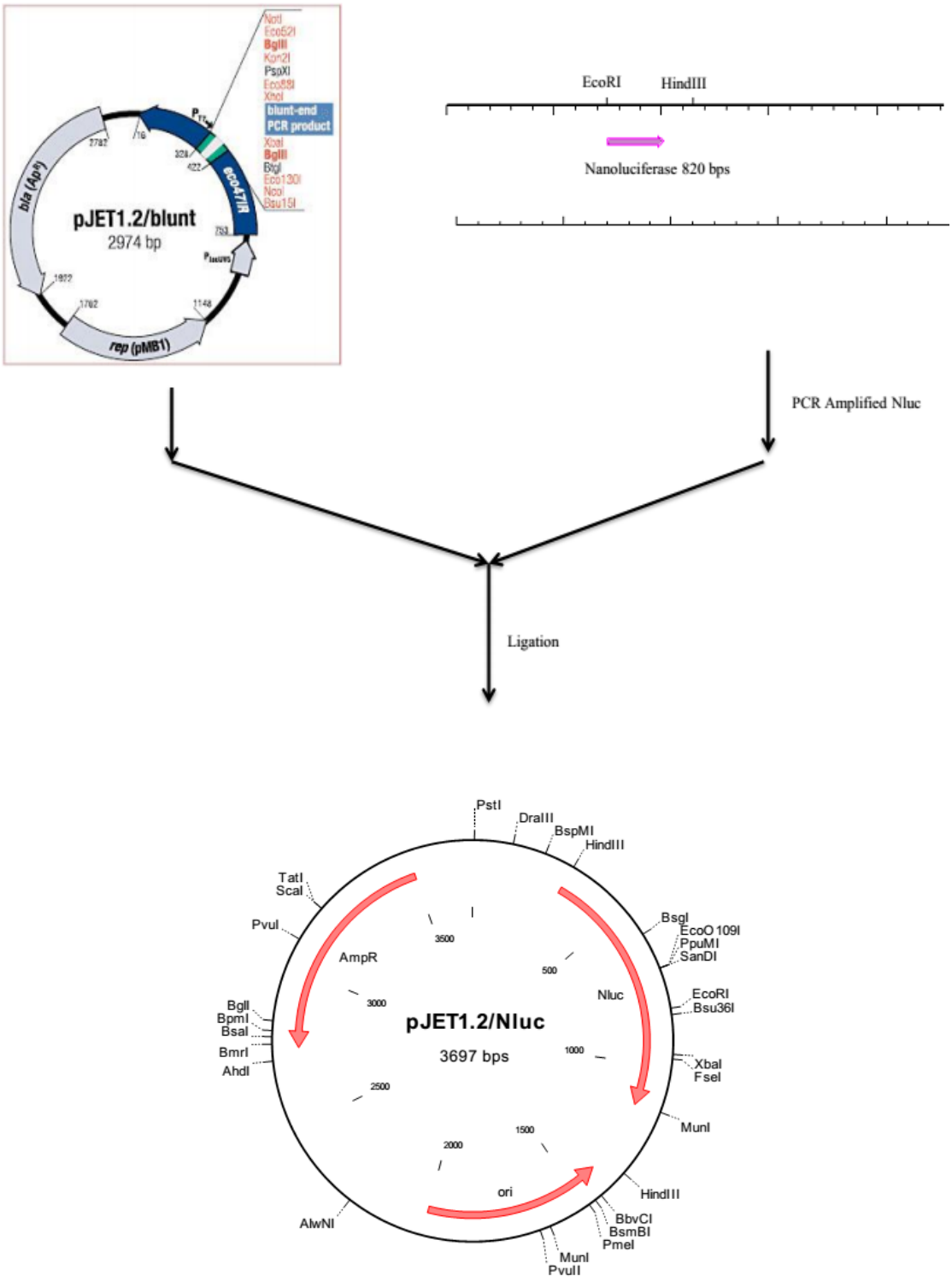

Figure 3.5.2. Construction of pJET1.2/Nluc Vector

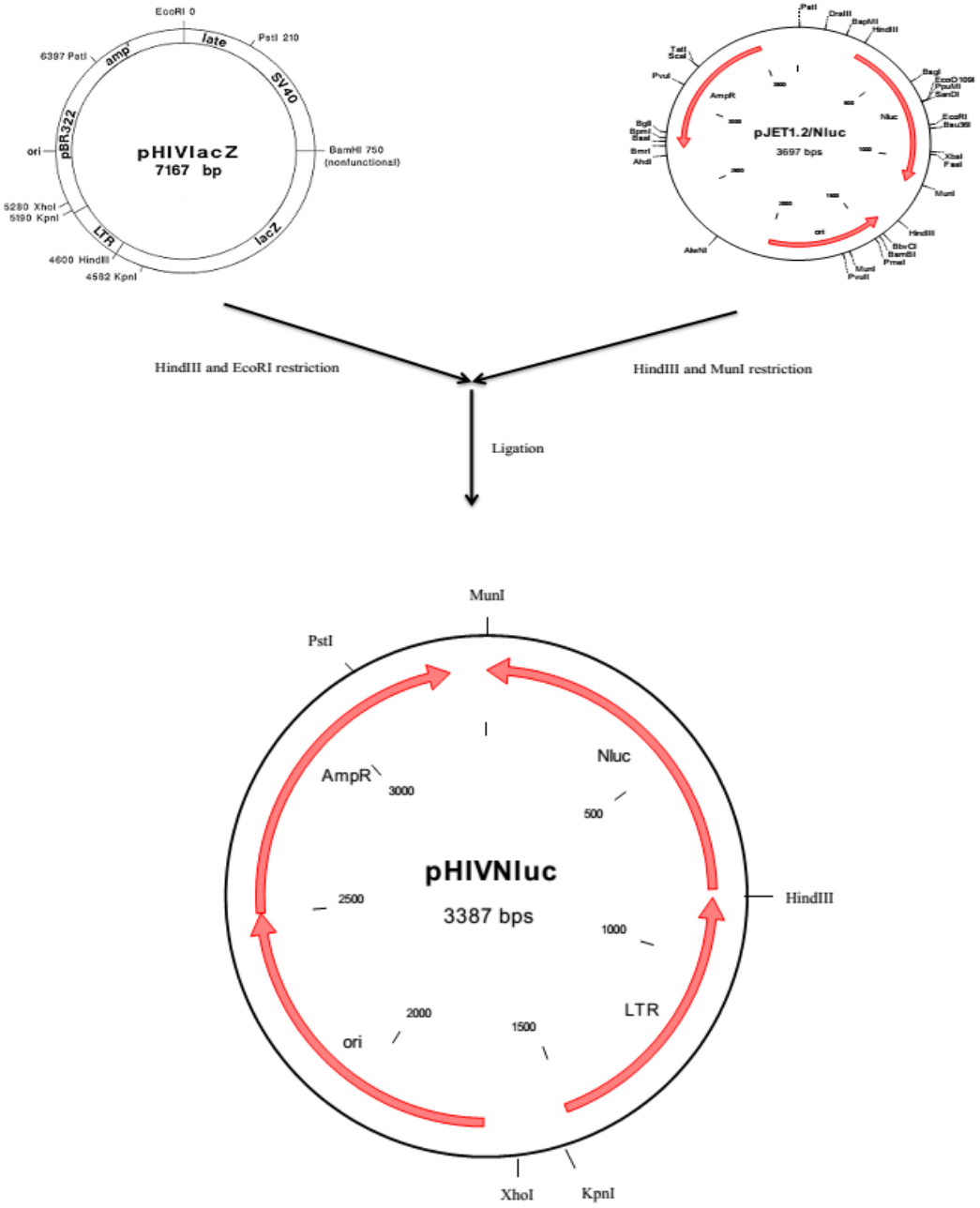

Figure 3.5.3. Construction of pHIVNluc Vector

3.6. Determination of Luciferase Expression

To detect the effect of the $SLPI_{agm}$ on the NF-kB promoter, 293 (HEK) and CV1 cells were transfected with pbudCE4.1, pNL-NF-kB and co-transfected with pNL-NFkB and pbudCE4.1_ $SLPI_{agm}$, pNL-NFkB and pbudCE4.1_tat plasmids. All assays contained blank, negative control and test samples. $SLPI_{agm}$ decreased the expression of luciferase for each cell lines. However, when HIV-1 tat gene is present in CV1 cell line, the expression of luciferase is decreased. Luminometrical values for each sample were shown in Figure 3.6.1.

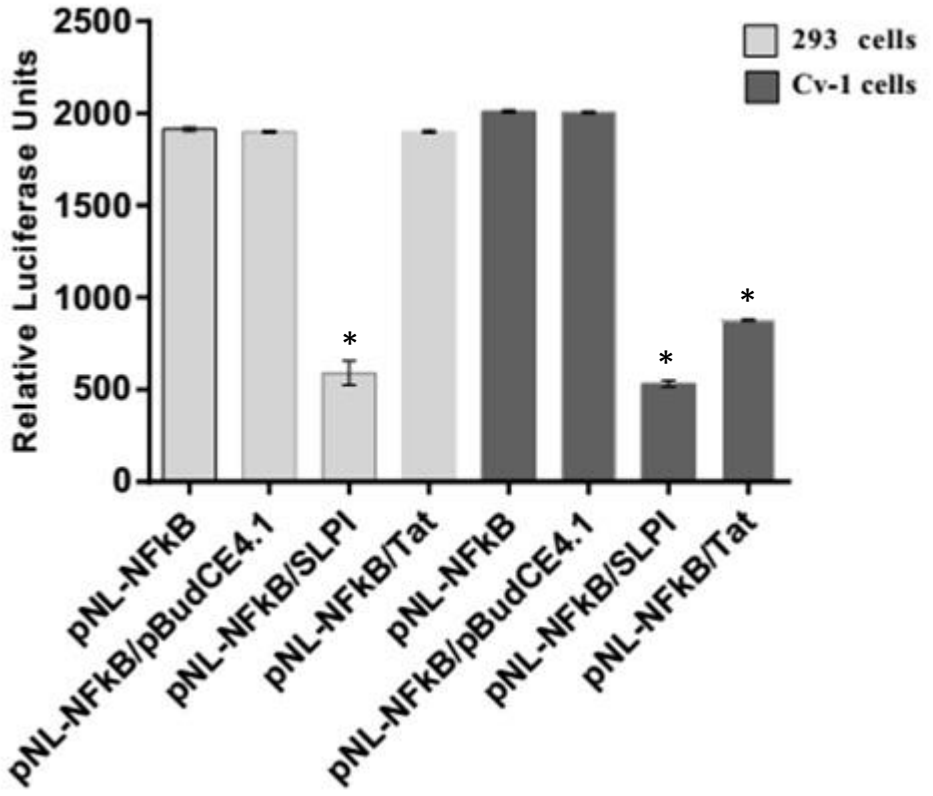

Figure 3.6.1. Luciferase expression level depend upon the NF-kB promoter in each sample (*:$p<0.05$).

In addition, to detect the effect of the $SLPI_{agm}$ on the HIV LTR promoter, 293 (HEK), HeLa, CV1, Vero and COS7 cells were transfected with pHIVNluc and co-transfected with pHIVNluc/pBudCE4.1 and pHIVNluc/tat plasmids. All assays contained blank, negative control and test samples. The expression of luciferase was decreased in pHIVNluc/ $SLPI_{agm}$ samples. While, in the presence of the *tat*, luciferase expression was increased in human cell lines, it was decreased in AGM cell lines.. Luminometrical values for each sample were shown in Figure 3.6.2.

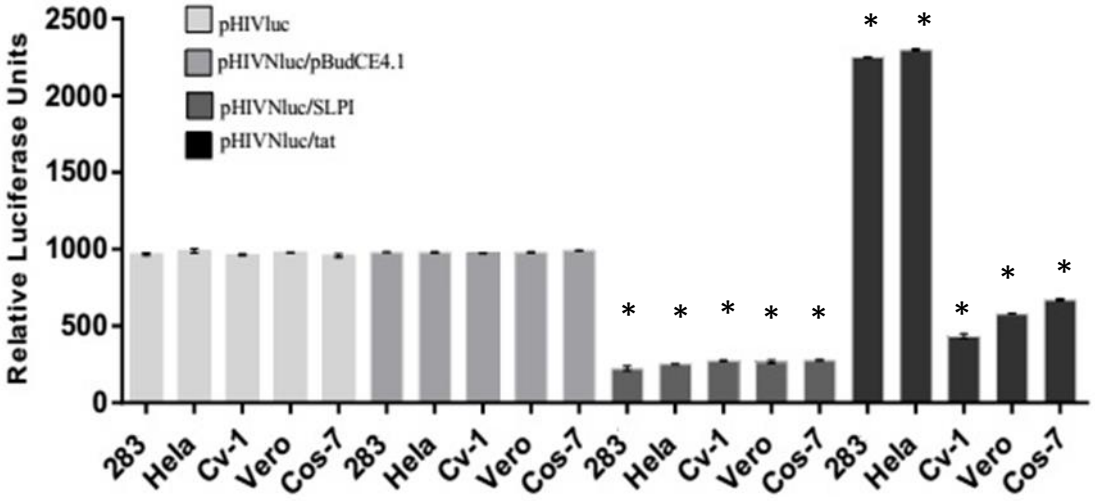

Figure 3.6.2. Luciferase expression level depend upon the HIV LTR in each cell line (*:$p<0.05$).

3.7. Determination of SLPI, GAG and TAT Gene Expression in HIV-1 Infected 293 (HEK) and CV1 Cell Lines

Our data demonstrate that $SLPI_{agm}$ gene was highly expressed in HIV-1 infected CV1 cells that have been transfected with pNL4.3. However, there was no important change in the HIV-1 infected 293 (HEK) cells (Figure 3.7.1). On the other hand, gag and tat gene expression was expressed HIV-1 infected CV1 and 293 (HEK). However, there was no expression of GAG and TAT gene in non infected cells (Figure 3.7.2). In addition, our data demonstrate that SLPI, GAG and TAT gene products were at correct size and there was no primer dimer (Figure 3.7.3 and 3.7.4)

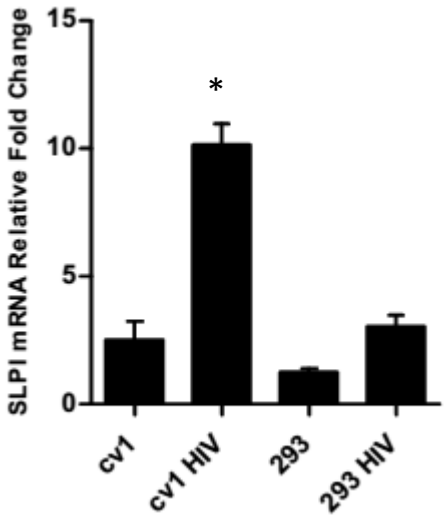

Figure 3.7.1. $SLPI_{agm}$ gene expression level in HIV-1 infected 293 (HEK) and CV1 cells (*:$p<0.05$).

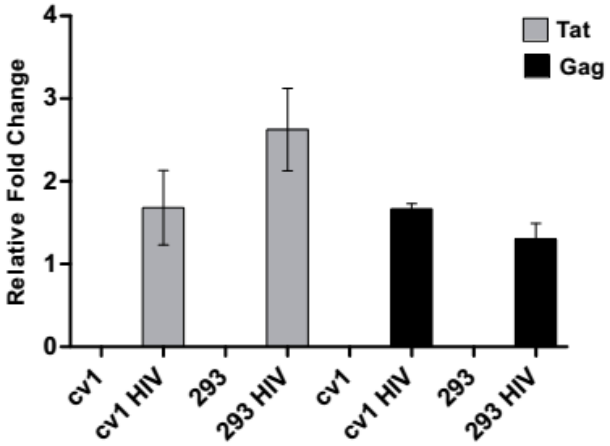

Figure 3.7.2. GAG and TAT gene expression level in HIV-1 infected 293 (HEK) and CV1 cells.

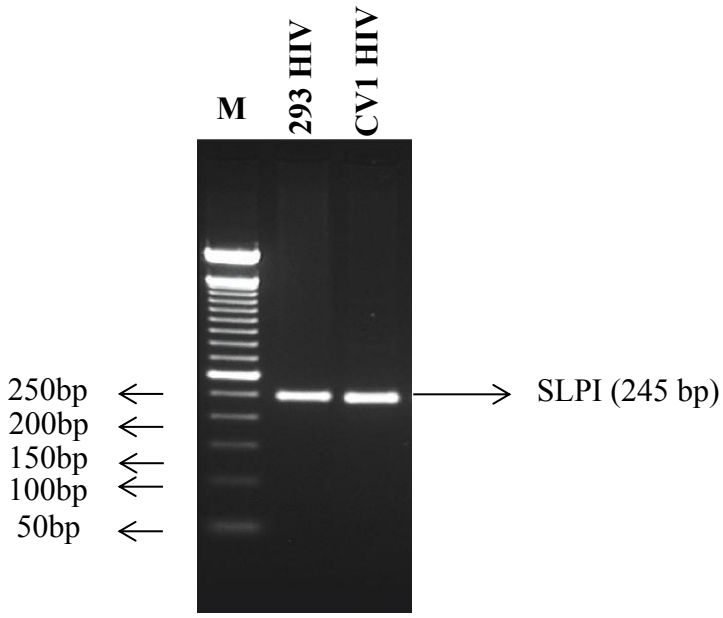

Figure 3.7.3. Agarose gel analysis of SLPI gene.

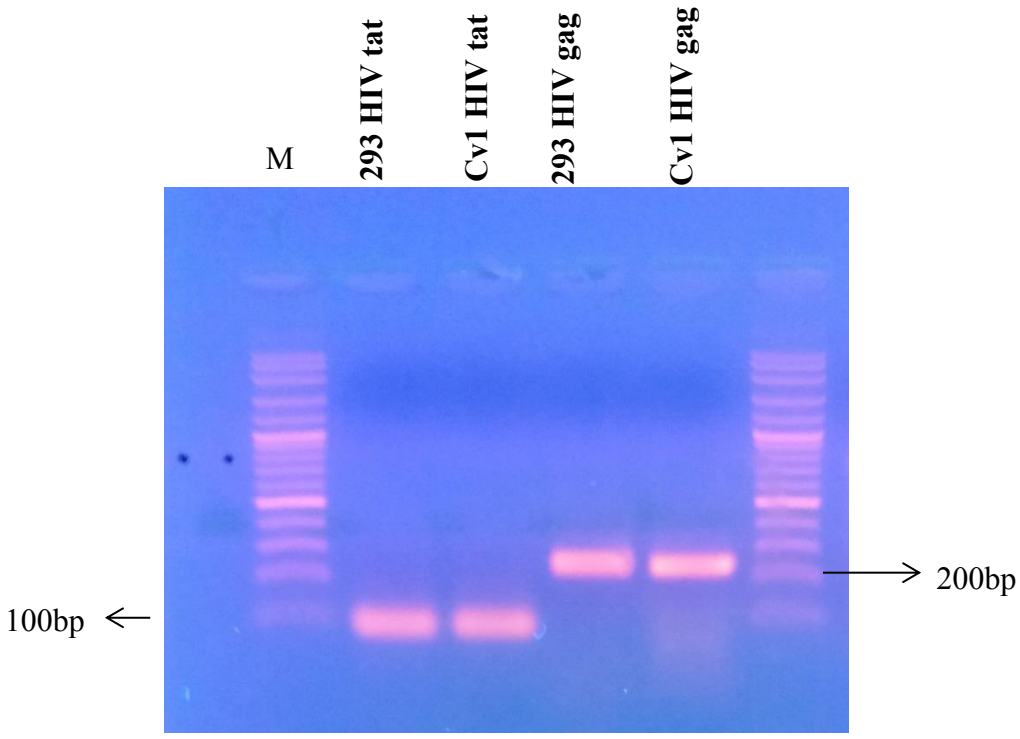

Figure 3.7.4. Agarose gel analysis of GAG and TAT gene.

3.8. Determination of HIV-1 p24 Antigen with ELISA

We initially constructed p24 antigen standard curve to detect the p24 antigen concentrations (Figure 3.8.1). Our data demonstrate that p24 antigen level was high in the HIV-1 infected 293 (HEK) and CV1 cells. On the other hand, p24 antigen level was low in HIV-1 infected and $SLPI_{agm}$ overexpressed 293 (HEK) and CV1 cells (Figure 3.8.2)

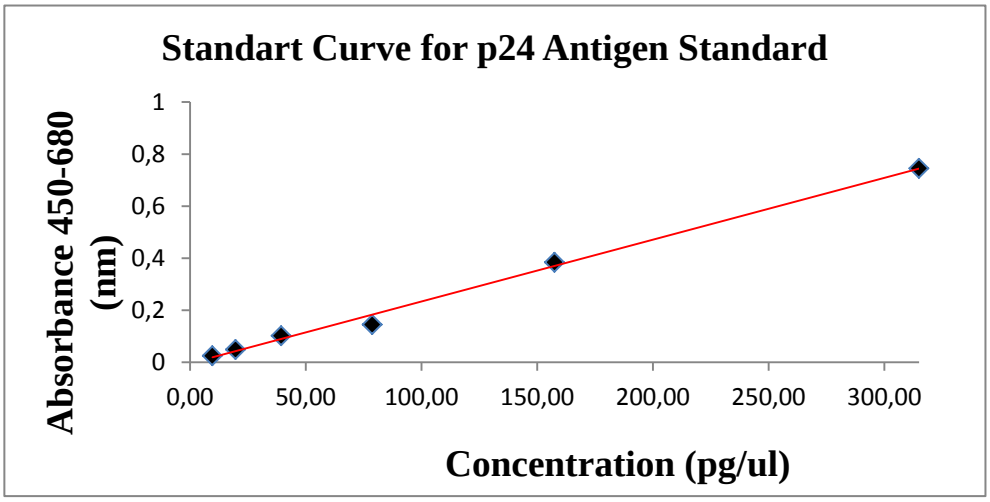

Figure 3.8.1. P24 Antigen Standard Curve

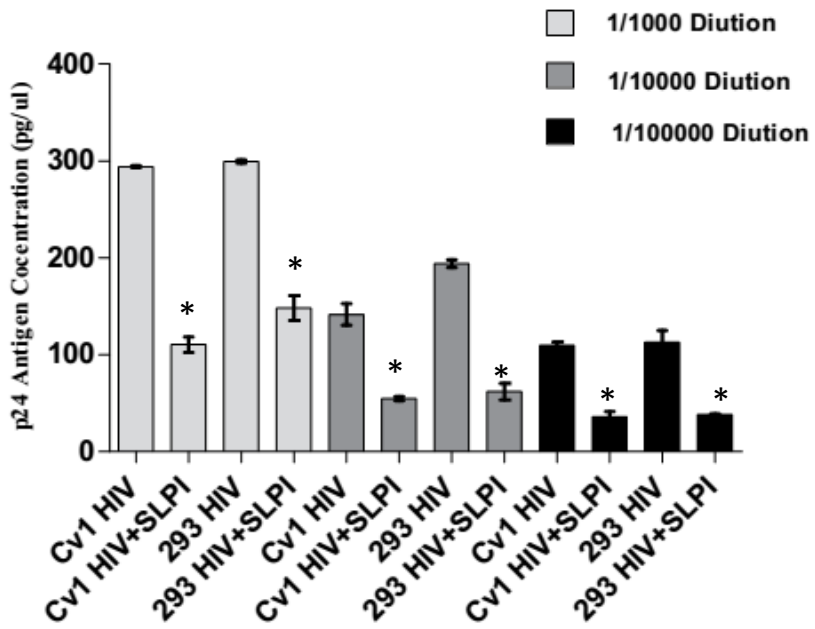

Figure 3.8.2. Concentrations of HIV-1 p24 antigen (*:p<0.05).

CHAPTER 4

DISCUSSION

We aimed in this study to research effect of HIV-1 tat gene on SLPI expression in African Green Monkey (AGM) and human cells and effect of SLPI on NF-kB and HIV LTR promoter. It has been shown in previous studies that Old World Monkeys (OWM) are resistant to HIV-1. Although resistance of OWM to HIV-1 seems encouraging for HIV-1 studies, HIV-1 related studies became difficult since these monkeys completely eliminate the infection after a while being infected with HIV-1 during these studies (Saito and Akari, 2013). It has been shown in publications that these monkeys were infected with HIV-1 only in case that immune system was weakened (Hatziioannou et al., 2014) . In previous studies, it has been shown that there are some factors which prevent HIV-1 replication before integration of HIV-1 genome into cell genome. These proteins were generally found in studies carried out on OWM. Some of them are Trim5 alpha, APOBEC and Fv4 proteins. Studies show that especially in high dosage HIV-1 infections of trim5 alpha, that protein is saturated. Nevertheless, elimination of the infection by OWM shows that there are other protein(s) except these restriction factors (Greene et al. 2008; Towers and Goff, 2003; Bishop et al., 2006; Bannert et al. 2006; Best et al., 1996; Kaiser et al., 2007).

We used African Green Monkey cells which are among OWM and human cells in our study. HIV-1 tat gene is a regulator protein for HIV-1 replication. Furthermore, in our study, we transfected HIV-1 tat gene to African Green Monkey and human cells and we ensured its expression by these cells. Since it would be negative in terms of cell economy for the cell to produce excessive protein against that genome after transfecting the entire gene of HIV-1 to the cells, we preferred to use only HIV-1 tat gene in this study. At the same time, we transfected HIV-1 tat gene to these cells and aimed to observe modifications to be occurred against HIV-1 tat. In our study, we observed that while SLPI expression increases in African Green Monkey cells in presence of HIV-1, there was no such increase in human cells. Furthermore, SLPI was high expressed in the HIV-1 infected CV1 cells. That condition is quite important in terms of our hypothesis. One of main reasons of that condition may be resistance of African green monkeys to

HIV-1. To explain more clearly, we may say that HIV-1 *tat* increases expression of SLPI protein against that protein after being integrated into monkey cells and being expressed. SLPI is among proteins in relation with the immune system at size 11.7 kilodaltons. SLPI was first found in chronic lung and cystic fibrosis patients. Later, it has been shown in studies that it has anti-bacterial and anti-fungal effects. Furthermore, it has been shown that it plays role in wound healing and prevents orally HIV-1 infection. It is very important in terms of our study that SLPI has such effect against HIV-1. However, studies show that the protein prevents HIV-1 replications before its integration into cell genome. It is unknown whether it has that effect or not following integration. We showed in our study that there is an increase in SLPI expression in presence of HIV-1 tat gene. That conclusion showed us that SLPI may have an eventual effect following integration of HIV-1 genome into the cell. HIV-1 LTR area is the area having vital importance for HIV-1 replication where both transcription factors of the cell and HIV-1 are connected. Furthermore, it has been shown in previous studies that HIV-1 LTR area has sp1, NF-kB and TAR connection points. NF-kB is among factors which ensure initiation of immune response by being expressed in cells infected by mitogens. Previous studies showed that SLPI protein causes suppression of NF-kB promoter. These results may show us that there may be a possible connection between HIV-1 LTR and SLPI protein.

During studies we have carried out later, we researched luciferase expression dependent on NF-kB promoter and HIV-1 LTR in presence of HIV-1 tat and $SLPI_{agm}$ in African Green Monkey cells and human cells in order to research whether there is a connection between HIV-1 LTR and SLPI or not. We set forth in this study as is in previous studies that $SLPI_{agm}$ protein suppresses NF-kB promoter. Furthermore, we observed that NF-kB promoter is suppressed in presence of HIV-1 tat gene in monkey cells. That result proved that $SLPI_{agm}$ expression increases in presence of HIV-1 tat. We observed that luciferase expression decreases both in presence of $SLPI_{agm}$ and HIV-1 tat gene in monkey cells in HIV-1 LTR dependent expression. That result shows us that SLPI suppresses HIV LTR. Furthermore, decrease of luciferase expression in presence of HIV-1 *tat* proves our former results. Once again, decrease of luciferase expression in human cells only in presence of SLPI proves our former results. Relation between $SLPI_{agm}$ and HIV LTR may be from the NK-kB connection point available at HIV-1

LTR area. These results may be explained by $SLPI_{agm}$ suppressing HIV-1 LTR by connecting to the NF-kB connection point available at HIV-1 LTR area.

HIV-1 p24 antigen level was low in HIV-1 infected and SLPI over expressed 293 (HEK) and CV1 cells compare to only HIV-1 infected 293 (HEK) and CV1 cells. Furthermore, $SLPI_{agm}$ suppressed the luciferase expression depend on HIV-1 LTR. Therefore, $SLPI_{agm}$ may decrease the HIV-1 replication by suppressing HIV-1 LTR promoter.

In future, more comprehensive studies to be carried out in relation with SLPI and HIV-1 may clearly set forth whether SLPI prevents or not HIV-1 infection after HIV-1 genome is integrated into the cell genome. Thus, it may set light to studies to be carried out in this field.

CHAPTER 5

CONCLUSION

In this study, we researched firstly SLPI protein expression in African Green Monkey and human cells in presence of HIV-1 tat gene. According to QRT-PCR results, we observed that HIV-1 tat gene increases SLPI expression in African Green Monkey cells. However, there was no such increase in human cells. According to the western blot analysis we carried out later, we proved that SLPI protein increases in presence of HIV-1 in African Green Monkeys. However, we observed that there is no such modification in expression of SLPI protein in human cells. Western blot results proved our QRT-PCR results that we carried out previously.

Later, we first cloned luciferase gene at HIV-1 LTR vector in order to research effect of $SLPI_{agm}$ on NF-kB and HIV-1 LTR and carried out the luciferase experiment. According to results of luciferase experiment, we observed that $SLPI_{agm}$ suppresses NF-kB promoter. Furthermore, we observed that NF-kB promoter is suppressed in presence of HIV-1 tat gene in African Green Monkey cells; however, there is no such effect on human cells. That result proved our former results.

When we carried out the luciferase experiment for HIV-1 LTR, we observed that HIV-1 LTR dependent luciferase expression decreases in African Green Monkey cells both in presence of SLPI and HIV-1 tat gene. However, we saw that such effect is in question only in presence of $SLPI_{agm}$ in human cells. These results show that SLPI expression increases in presence of HIV-1 tat gene in African Green Monkey cells and suppresses HIV-1 LTR. Furthermore, these results prove former QRT-PCR and Western Blot results.

When we infected 293 (HEK) and CV1 cell lines with HIV-1 molecular clone, SLPI mRNA expression level in infected cv1 cells was high compare to non-infected cv1 cells. However, SLPI mRNA expression level was not changed between infected and non-infected 293 (HEK) cells.

The overexpression of $SLPI_{agm}$ decreased the level of p24 antigen in HIV-1 infected CV1 and 293(HEK) cell lines.

Consequently, as a response to the question whether there is other protein(s) or not apart from integration of HIV-1 replication into the cell genome, SLPI may be one of these proteins. In future, more detailed studies to be carried out in this field may reveal that SLPI protein may be the eventual enemy against HIV-1 infection.

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APPENDIX A

BUFFERS AND STOCK SOLUTIONS

Luria Bertani (LB) broth, per liter

10 g tryptone, 5 g yeast extract, 5 g NaCl and dH2O up to 1 L.

Luria Bertani (LB) agar, per liter

10 g tryptone, 5 g yeast extract, 5 g NaCl, 15g agar and dH2O up to 1 L.

X-GAL Stock Solution (50 mg/ml)

0.1 g x-gal in 2 ml N-N di-methyl formimide.

IPTG Stock Solution (0.1 M)

1.2 g IPTG was dissolved in 50 ml of deionized water

SOC Medium, per 100 ml

2 g Tryptone, 0.5 g Yeast Extract, 1 ml 1M NaCl, 0.25 ml 1M KCl, 1 ml 2M Mg2+ Stock, 1 ml 2M Glucose were dissolved in dH2O up to 100 ml.

SOB Medium, per 100 ml

2 g Tryptone, 0.5 g Yeast Extract, 1 ml 1M NaCl, 0.02 g KCl, 1 ml 1M MgCl, 1 ml 1M MgSO4 were dissolved in deionized dH2O up to 100 ml.

10% Ammonium Persulfate

0.1 g ammonium persulfate was dissolved in 1.0 ml dH2O.

50X TAE Electrophoresis Buffer

242 g Tris base and 37.2 g Na2EDTA (2H2O) was dissolved in 900 ml deionized water. After 57.1 ml glacial acetic acid was added, the volume was adjusted to 1 liter with deionized water.

Ethidium Bromide Stock Solution (10 Mg/Ml)

0.2g ethidium bromide (EtBr) was dissolved in 20 ml dH2O. It was mixed well and stored at room temperature in dark.

Tris-HCL buffer (50 mM), pH 8

0.6 g tris base dissolved in 100 ml water and then pH was adjusted with HCL.

10X SDS Running Buffer

30.3 g tris base, 144 g glycine, 10 g SDS were dissolved in 1 L water and then pH was adjusted to 8.3.

Blocking Buffer

5% non-fat dry milk powder was dissolved in PBS and 0.05% Tween 20 was added.

Transfer Buffer

39 mM glycine, 48 mM tris base, 0.037% SDS, 20% methanol were dissolved in water and then pH was adjusted to 8.3