Functional Analysis of Simian Immunodeficiency Virus Vpx Protein

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Declaration

The research work embodied in this thesis entitled **"Functional Analysis of Simian Immunodeficiency Virus Vpx Protein"**, has been carried out by me at the Centre for DNA Fingerprinting and Diagnostics, Hyderabad, under the guidance of Dr. S. Mahalingam. I hereby declare that this work is original and has not been submitted in part or full for any other degree or diploma of any other university or institution.

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Certificate

This is to certify that this thesis entitled, "**Functional Analysis of Simian Immunodeficiency Virus Vpx Protein**", submitted by Mr. P.Rajendra Kumar, for the degree of Doctor of Philosophy to the University of Hyderabad is based on the work carried out by him at the Centre for DNA Fingerprinting and Diagnostics, Hyderabad. This work is original and has not been submitted for any diploma or degree of any other university or institution.

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P. Rajendra kumar

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Abbreviations and symbols :

Ab	Antibody
AIDS	Acquired immunodeficiency syndrome
ARM	Arginine-rich binding motif
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
CA	Capsid
CCR5	C-C chemokine receptor 5
CD	Cluster of differentiation
CDK	Cyclin dependent kinase
cDNA	Complementary DNA
CXCR4	CXC chemokine receptor 4
DMEM	Dulbecco's modified Eagle medium
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
ERK-2	Extra cellular signal regulated kinase-2
Env	Envelope protein
FBS	Fetal bovine serum
GFP	Green fluorescence protein
GST	Glutathione-S-transferase
HIV	Human immunodeficiency virus
hr	Hours
HRP	Horse radish peroxidase
IN	Integrase
IPTG	Isopropyl-β -D-thiogalactopyranoside
kDa	Kilodalton
kb	Kilobases
LTR	Long terminal repeat

MA	Matrix
MAPK	Mitogen activated protein kinase
MHC I	Major histocompatibility complex class I
mCi	Milli curie
MHC II	Major histocompatibility complex class II
mg	Milligram
ml	Milliliter
µg	Microgram
min	Minutes
NC	Nucleocapsid
Nef	Negative factor
NIK	NF- B inducing kinase
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenyl methyl sulfonyl fluoride
PR	Protease
SDS	Sodium dodecyl sulpahte
SIV	Simian immunodeficiency virus
RT	Reverse transcriptase
TBP	TATA binding protein
Tat	Transactivator protein of HIV
VIF	Virus infectivity factor
VPR	Viral protein R
VPU	Viral protein U
VPX	Viral protein X

Chapter One

Introduction

Emerging infectious diseases represent substantial threats to global health and Acquired Immunodeficiency Syndrome (AIDS) ranks as one of the most important infectious diseases that humans are facing in the 21st century. Since its clinical description less than two decades ago, AIDS has resulted in the deaths of more than 20 million people worldwide. Human immunodeficiency virus type-1(HIV-1) and type-2 (HIV-2), the etiological agents for AIDS, have infected more than 50 million individuals worldwide, and the new infections increase at the rate of 6 million per year. Although sub-Saharan Africa remains the global epicenter, rates of infection have increased in recent times in the former Soviet Union and parts of south and south-east Asia, including India and China. Combination of anti-retroviral therapy has afforded many people clinical relief but vast majority of the infected people worldwide do not have access to these agents.

The AIDS viruses HIV-1, HIV-2 and the closely related simian immunodeficiency viruses (SIVs) belong to lentivirus subfamily of retroviruses. These viruses have remarkable properties of insidious disease induction, persistence, latency, variation, recombination and escape from immune and drug pressures. HIV-1 and HIV-2 are the result of zoonotic transmission of SIVcpz in chimpanzees (*Pan troglodytes troglodytes*) from West central Africa and SIVsm in sooty mangabeys (*Cercocebus atys*) from West Africa respectively. At least three different zoonotic jumps from chimpanzees into humans led to the disproportionate spread of HIV-1 groups M, O and N. In addition humans have apparently picked up as many as seven lineages of viruses from sooty mangabeys resulting in HIV-2 subtypes A through G. HIV-1, HIV-2 and SIV have a ~10kb RNA genome, and carry three structural genes (*gag*, *pol* and *env*) and two regulatory genes (*rev* and *tat*) that are essential for replication. These viruses also have accessory genes that are not essential for

replication *in vitro*, but can dramatically alter the course and severity of infection, replication and disease progression *in vivo*. *Vif, vpr and nef* are present in HIV-1, HIV-2 and related SIV where as *vpu* is present only in HIV-1 and related SIVcpz and SIVgsn. In contrast, Vpx is present only in HIV-2 and related SIVsm, SIVrcm, and SIVmac. Containment of AIDS epidemic will require an effective vaccine against HIV infection. Lack of animal model has hampered the vaccine development, even though far from perfect non-human primates offer the only animal model due to similarity in physiology and natural course of infection. SIV naturally infect a variety of non-human primates. Although SIV share many structural and biological properties with human immunodeficiency viruses, they do not seem to induce AIDS in their natural hosts. In contrast, SIV from sooty mangabeys (SIVsm) induces an immunodeficiency syndrome very similar to AIDS when it is experimentally inoculated into Asian monkey species such as rhesus macaques. Non-human primate models continue to provide an important tool for assessing the extent of protective immunity induced by various immunization strategies.

A critical step in the process of retrovirus infection is the transfer of viral DNA into the nucleus of the infected cells. Lentiviruses like HIV/SIV are capable of infecting non-dividing cells such as terminally differentiated macrophages and memory T-cells, which are important for *in vivo* viral dissemination and persistence. In contrast, prototypic retroviruses do not replicate efficiently in non-dividing cells. After viral entry into the cell, genomic HIV/SIV RNA is reverse transcribed into linear double-stranded DNA, which remains associated with a nucleoprotein complex called preintegration complex (PIC). The viral PICs are then imported into the nucleus through the nuclear pore complex (NPC) via an active mechanism within four to six hours after infection. One cis-acting element, central DNA flap and at least three

proteins namely integrase, Gag matrix protein and Vpr protein have been identified as possible mediators of the nuclear import of the HIV-1 PIC. Interestingly HIV-2/SIVsm/SIVmac contains a *vpr* gene as well as evolutionarily related *vpx* gene. Vpr induces cell cycle arrest at G2 stage whereas Vpx was found to be the major determinant involved in the nuclear transport of PIC. Vpx is also essential for efficient *in vivo* dissemination and spread of SIVsm following mucosal and intravenous infection of macaques. Vpx mutant SIVsm is significantly reduced in its ability to replicate in non-dividing target cells such as monocyte derived macaque macrophages. Vpx is packaged into the viral particles via its interaction with structural protein Gag. Based on such late expression during virus production and early availability during initial infection, it has been proposed that Vpx is involved in the efficient import of viral DNA into the nuclear compartment of non-dividing target cells. But the domains required for Vpx nuclear import and for the efficient virus replication in non-dividing cells have so far not been reported. Also, the mechanism by which Vpx regulates the nuclear import of HIV-2/SIVsm remains unknown.

The present study was designed to address the following issues:

1. Identification and characterization of signal(s) involved in Vpx nuclear localization.
2. Elucidating the mechanism(s) that regulate the nuclear transport of Vpx.
3. To define the signal(s) and the mechanism(s) of Vpx packaging into virus particles.

Chapter Two

Review of literature

Since its discovery, HIV has drawn more attention from scientists than any other infectious disease. AIDS was first identified in 1981 in U.S when Centre for Disease Control and Prevention (CDC), received reports where a group of previously healthy homosexual men displayed unusual infections, like the opportunistic fungal pathogen pneumocystis carinii, which causes pneumonia, Toxoplasma gondii encephalitis and Kaposi's sarcoma, a rare tumor of blood vessel tissue associated with ageing. A laboratory finding common to all such individuals was marked depletion of the CD4+ T lymphocyte population in their peripheral blood (Moss and Bacchetti, 1989). In 1982, CDC proposed that this new disorder be called as acquired immunodeficiency syndrome (AIDS). Though initially identified in homosexual men, the unidentified agent was also observed in other groups, including users of intravenous drugs, hemophiliacs, blood transfusion recipients and eventually in infants of mothers with disease. These observations suggested that the causative agent is transferred through the transfer of blood from infected persons to healthy people.

In 1983 Luc Montagnier's group at Pasteur institute isolated a retrovirus from the lymph node biopsy of a patient with AIDS (Barre-sinoussi *et al*., 1983). The virus showed reverse transcriptase activity and has morphology of a retrovirus, subsequently R.C.Gallo at the National institutes of health (NIH) isolated a retrovirus from an AIDS patient, which they named human T-lymphotrophic virus-III (HTLV-III) (Popovic *et al*., 1984). Based on its morphological and genetic characterization, in 1986 the virus has been named as human immunodeficiency virus or HIV by the international committee on taxonomy of viruses (Coffin *et al*., 1986).

2.1 Global impact of AIDS.

Over 20 years since it was identified, HIV/AIDS epidemic has continued to exceed all expectations in severity and scale of impact. Globally, the epidemic continues to exact a devastating toll on individuals and families.

In sub-Saharan Africa, it is erasing decades of health, economic and social progress, reducing the life expectancy by decades, slowing the economic growth, deepening poverty and contributing to and exacerbating chronic food shortage.

According to AIDS epidemic update in 2005 there are close to five million new HIV infections worldwide annually, out of which 3.2 million are in sub-Saharan Africa alone. In the same year 3 million people died of AIDS–related diseases, more than half a million (570 000) were children. Today the total number of people living with HIV stands at 40.3 million, double the number (19.9 million) in 1995. (Fig.1) shows the number of adults and children living with AIDS.

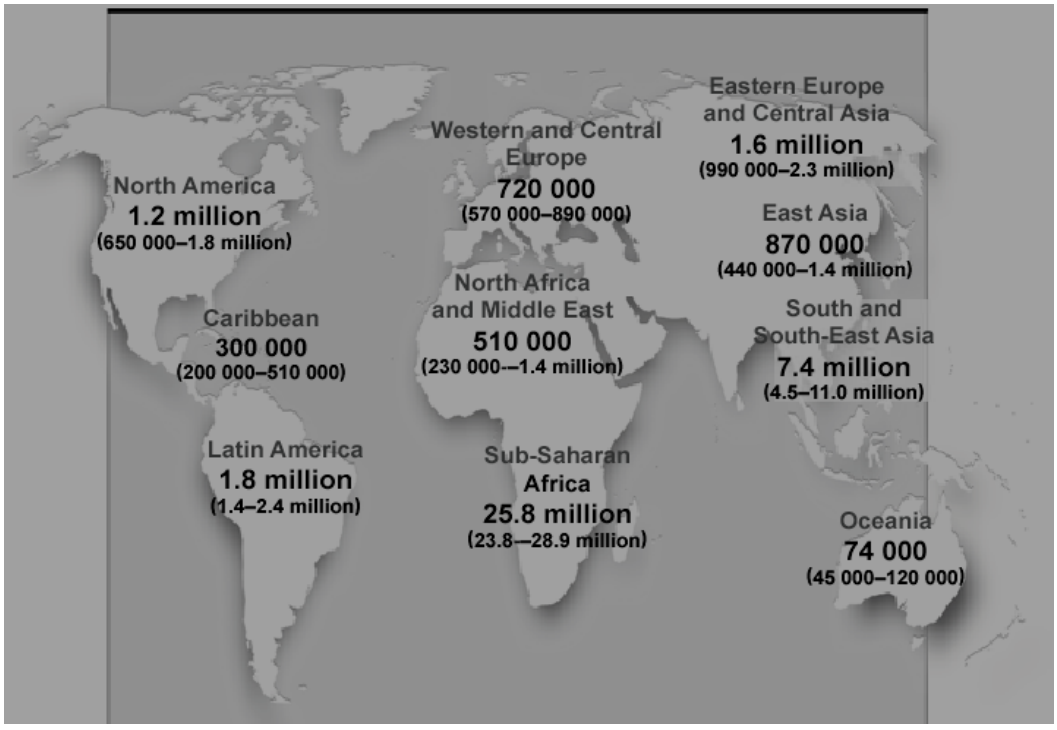


Figure 1: Estimated number of adults and children living with HIV in 2005 (UNAIDS, AIDS epidemic update 2005)

The epidemic's impact on women and girls is especially marked. Most women in the hardest-hit countries face heavy economic, legal, cultural and social disadvantages which increase their vulnerability to the epidemics impact. The number of people living with HIV has increased all over the world in the past two years. Sub-Saharan Africa remains the epicenter, and is home to 25.8 million people living with HIV. Two thirds of all people living with HIV are in sub-Saharan Africa, as are 77% of all women with HIV. The epidemics in Asia and Oceania are increasing, particularly in China, Papua New Guinea and Vietnam. There are alarming signs that other countries including Pakistan and Indonesia could be on the verge of serious epidemics. Across Asia, the epidemics are propelled by combinations of injecting drug use and commercial sex. Only a handful of countries are making serious–enough efforts to introduce programmes focusing on these risky behaviors on the scale required. In India the epidemic has reached an alarming proportion with the number of HIV infected patients being next to South Africa.

AIDS responses have grown and improved over the past decade. But they still do not match the scale or the pace of a steadily worsening epidemic. In past two years access to retroviral therapy has improved the quality of HIV infected individual's life markedly. Bringing AIDS under control will require tackling with greater resolve the underlying factors that fuel these epidemics-including societal inequalities and injustices. It will require overcoming the still serious barriers to access that take the form of stigma, discrimination, gender inequality and other human rights violations.

2.2 Origin and Diversity.

Human immunodeficiency viruses HIV-1 and HIV-2 are the etiological agents for AIDS in humans. HIV-1 has spread to most parts of the world, while HIV-2 has remained largely restricted to West Africa and India (Gallo and Montagnier, 2003; Schim van der loeff and Aaby, 1999). Genetic variation is

common to all RNA viruses but has been best characterized for HIV-1. The extensive heterogeneity observed in the worldwide epidemic of HIV-1 originates from the rapid viral turnover (10^{10} viral particles/day) and the high rate of incorrect nucleotide substitutions during HIV reverse transcription (10^{-4}/nt) in the absence of proof reading mechanisms (Kevlen *et al*., 1997; Drosopoulos *et al*., 1998). HIV continually evolves and migrates through individual hosts, overcoming barriers to transmission, avoiding different immune responses, and resisting various anti-retroviral regimens (Sharp *et al*., 1999; Brandes and Walker, 2003; Rowland-jones, 2003, Apetrei *et al*, 2004; Spira *et al*., 2003).

2.2.1 Zoonotic transmission of Lentiviruses.

HIV-1, HIV-2 and the closely related simian immunodeficiency viruses (SIV) belong to lentivirus subfamily of retroviruses. Humans are exposed to a plethora of primate lentiviruses through hunting and handling of primate bush meat in Central Africa (Peeters *et al*., 2002). Apart from HIV-1 and HIV-2 many other viruses have also made leap from simians to humans like the monkey pox virus (Meyer *et al*., 1991), simian T-cell leukemia virus/human T cell leukemia virus (Voevodin *et al*., 1997 Slattery *et al*.,1999) and simian foamy virus (Heneine *et al*., 1998; Callahan *et al*., 1999).

HIV-1 and HIV-2 are result of zoonotic transmission of SIVcpz in chimpanzees (*pan troglodytes troglodytes*) from west central Africa (Gao *et al*., 1999) and SIVsm in sooty mangabeys (*cercocebus atys*) from West Africa (Gao *et al*., 1992) respectively. HIV-1 strains fall into three highly divergent groups, major M group, a minor N group and outlier O group (Gurtler *et al*., 1994; Mauclere *et al*., 1997; Peeters *et al*., 1997; Simon *et al*., 1998) each of these are more closely related to SIVcpz *pan troglodytes troglodytes* (Fig.2) (Hahn *et al*., 2000; Sharp *et al*., 2001). These three sub groups are not each other closest relatives but instead were interspersed with SIVcpz within HIV-

1/SIVcpz radiation. This finding indicates that there had been three separate introductions of SIVcpz into human population. Out of these three groups, group M is the most widely spread. Molecular clock analysis showed that the origin of HIV-1 group M radiation was in 1930s. Korber *et al*., 2000 have estimated that HIV-1 M group viruses last shared a common ancestor between 1915 and 1941.

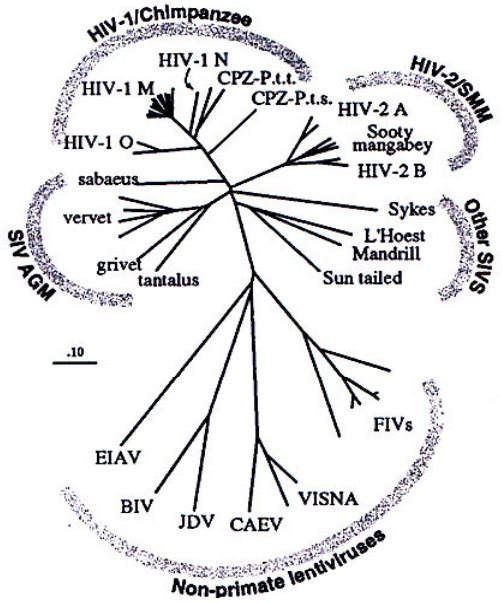


Figure 2: Phylogenetic relationship of primate lentivirus (Hahn *et al*., 2000)

On the basis of phylogenetic analysis of numerous isolates obtained from diverse geographic origins, HIV is divided into groups, types, sub-types, sub-sub types, circulating recombinant forms and unique recombinant forms. Currently nine sub-types of HIV-1 group M exist, they are A-D, F-H, J and K (Fig.3). Sub-types form clusters roughly equidistant from each other in phylogenetic trees (Apetrei *et al*., 2004; Spira *et al*., 2003; Robertson *et al*., 2000; Stebbing and Moyle, 2003). HIV-1 groups O and N, which are genetically very divergent from group M, represent less than 5 per cent of infections worldwide and have almost exclusively been detected in West Central Africa (Sharp *et al*., 2000; Roques *et al*., 2004; Simon *et al*., 1998; Peeters *et al*., 1997). Group O viruses have been identified mainly from

persons with epidemiological links to West Central Africa, mainly Cameroon and some neighboring countries. Whereas HIV-1 group N infection has been identified only in Cameroon, a country endemic for HIV-1 with all the major groups in co-circulation.

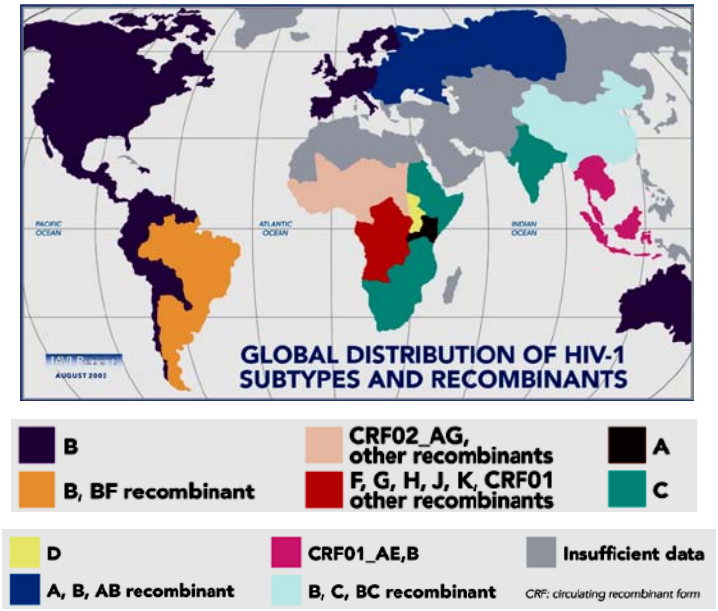


Figure 3: Geographical distribution of HIV-1 subtypes, and circulating recombinant forms (CRFs) in different parts of the world. Ten different epidemic patterns have been observed, as shown in different colors. (Data adopted from www.iavireport.org).

Epidemiology and molecular phylogeny of HIV-2 viruses are less well understood than those of the HIV-1. The diverse HIV-2 lineages are thought to have arisen due to several cross-species transfers from sooty mangabeys into humans, one for each subtype of HIV-2 (Apetrei *et al*., 2004; Damond *et al*., 2004). So far seven subtypes (A-G) of HIV-2 have been described with subtypes A and B being the predominant strains (Damond *et al*., 2004). The nucleotide and amino acid sequence diversity of HIV-2 viruses is greater than the diversity of the HIV-1 group M viruses but roughly equivalent to the diversity with in HIV-1/SIVcpz clade. The subtypes of HIV-2 are analogous to the groups (M, N and O) of HIV-1, in terms of the cross-species transfer events thought to have created them (Gao *et al*., 1994; Lemey *et al*., 2003; Damond *et al*., 2004).

2.3 Genomic organization of lentiviruses.

The common genomic structure for primate lentiviruses is LTR-*gag-pol-vif-vpr-tat-rev-env-nef*-LTR, but some lentiviruses have an additional gene *vpu* or *vpx* in the central part of the genome (Fig.4) (Baier *et al*., 1990; Beer *et al*., 1999; Courgnaud *et al*., 2003; Fomsgaard *et al*., 1991; Hirsch *et al*., 1993; Hirsch *et al*., 1999). HIV-2 related viruses like SIVsm and SIVmac, as well as SIVrcm and SIVmnd2 have a *vpx* gene upstream of the *vpr* gene (Beer *et al*., 2001; Chakrabarti *et al*., 1987; Clavel *et al*., 1986; Hirsch *et al*., 1989; Souquiere *et al*., 2001). Until recently, only the viruses belonging to the SIVcpz/HIV-1 lineage were known to carry an additional *vpu* gene upstream of *env* but now the presence of *vpu* has also been documented in SIVs from *Cercopithecus* species like SIVgsn from *C.nictitans* in Cameroon.

2.3.1 mRNA Splicing and Cellular Localization.

The primary HIV-1 transcript contains multiple splice donors (5' splice sites) and splice acceptors (3' splice sites), which can be processed to yield more than 30 alternative mRNAs (Schwartz *et al*., 1990). Many of the mRNAs are polycistronic i.e., they contain the open reading frame for more than one protein. Open reading frame choice is governed by the efficiency of the initiation codon and the proximity of the initiation codon to the 5' end of the mRNA (Schwartz *et al*., 1992). HIV-1 mRNAs fall into three size classes, unspliced, singly spliced and multiply spliced. The unspliced 9-kb primary transcript can be expressed to generate the Gag and Gag-Pol precursor proteins or be packaged into virions to serve as the genomic RNA. Incompletely spliced RNA's, use the splice donor site located nearest to the 5' end of the HIV RNA genome in combination with any of the splice acceptors located in the central region of the virus. These RNAs can potentially express Env, Vif, Vpu, Vpr, and the single-exon form of Tat. These heterogeneous mRNAs are 4-5 kb long and retain the second intron of HIV. Fully spliced

RNA's, have spliced out both the introns of HIV and have the potential to express Rev, Nef, and the second exon of Tat. These heterogeneous mRNAs do not require the expression of the Rev protein.

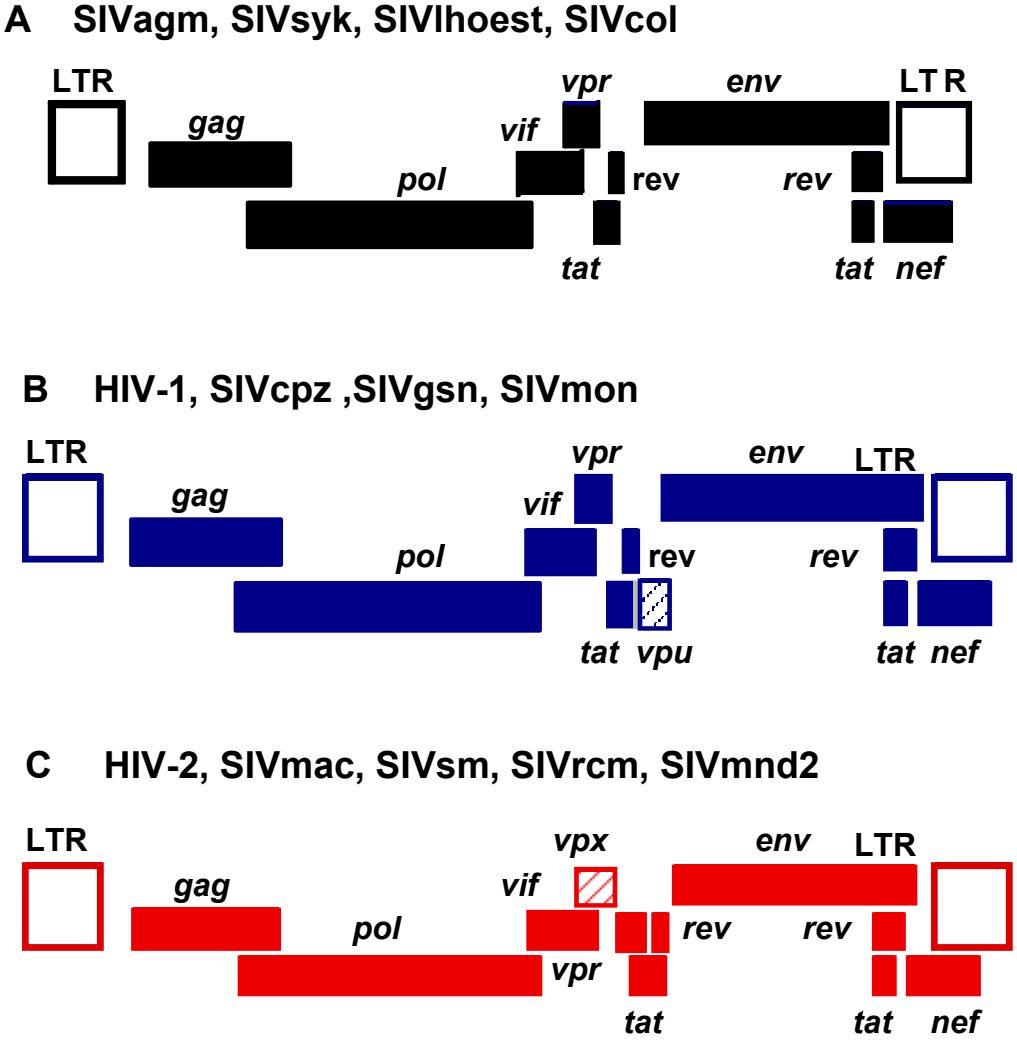


Figure 4: Genomic organization of primate lentiviruses

2.3.2 Structural proteins.

2.3.2.1 Gag.

HIV assembly is controlled primarily by the Gag protein, one of the three structural gene products encoded by all the retroviruses. Gag orchestrates assembly by recruiting all the building blocks required for the formation of a

fully infectious virion. Gag provides the principal driving force for virus assembly, as illustrated by the fact that Gag can efficiently form virus-like particles when expressed even in the absence of other viral proteins (Gheysen *et al*., 1989). Gag is often referred to as a precursor, because it is subject to cleavage by viral protease (PR), which yields internal mature structural proteins of the mature virion (Freed, 1998; Fuller et al., 1997; Wills and Craven, 1991). PR and other essential viral enzymes are brought into the virion as components of the Gag-pol polyprotein, which is produced by ribosomal frame shifting between overlapping Gag and Pol open reading frames. Three of the Gag cleavage products, matrix (MA), capsid (CA) and nucleocapsid (NC), are common to all retroviruses. Additionally, the Gag precursor of HIV/SIV possess a C-terminal domain called p6 that is unique to primate lentiviruses, as well as two spacer regions which separate CA from NC, and NC from p6 (Henderson *et al*., 1992; Mervis *et al*., 1988). The Gag precursor is synthesized on the cytosolic ribosomes (Tritel and Resh, 2000) and becomes co-translationally modified by the N-terminal attachment of a myristyl group, which increases its affinity for membranes (Bryant and Ratner, 1990; Gottlinger *et al*., 1989).

Immature HIV particles are non-infectious (Gottlinger *et al*., 1989; Kohl *et al*., 1988), because the palisade like shell of radially arranged, unprocessed Gag precursor molecules which forms immature capsid is too stable to permit its disassembly. This prevents uncoating after virus entry into a new target cell, which is essential to release the viral genome in a form that can be imported into the nucleus and integrated into the host genome. The immature capsid thus needs to undergo a maturation step that makes it metastable, and this process is initiated by the activation of PR. This leads to a series of proteolytic processing events at the boundaries within the Gag precursor, and is followed by drastic rearrangement of the liberated Gag domains. Only the MA domain remains associated with the viral lipid core, while the CA and NC

domains condense around the viral genome, yielding a characteristic conical core of the mature virion.

2.3.2.2 Matrix.

Matrix (MA) constitutes the N-terminal domain of the Gag precursor and remains intimately associated with the lipid envelope of the mature virion. During assembly, MA has established roles in the targeting of Gag to the plasma membrane and in the incorporation of the viral envelope (Env) glycoproteins into nascent particles. Additionally, MA has also been implicated in post-assembly steps of the viral replication cycle, particularly in non-dividing cells.

The membrane–targeting function of MA is dependent on the N-terminal attachment of a myristic moiety (Bryant and Ratner, 1990; Gottlinger *et al*., 1989; Spearman *et al*., 1994). This hydrophobic modification is crucial for the stable membrane association of the Gag precursor, and is essential for extra cellular particle formation and virus replication. In addition to the hydrophobic myristyl anchor, a charged N-proximal region also contributes to the membrane targeting ability of MA (Yuan *et al*., 1993; Zhou *et al*., 1994). This region harbors a cluster of conserved basic residues that can bind to acidic phospholipids *in vitro* (Zhou *et al*., 1994). In addition to its role in Gag membrane targeting, MA is essential for the incorporation of the Env glycoprotein spikes during virus assembly (Dorfman *et al*., 1994b; Yu *et al* 1992a). Localization of Env determines the site of budding in polarized cells (Owens *et al*., 1991) and in addition to a specific sorting signal in the cytoplasmic domain of the transmembrane domain (TM) of the Env, an intact MA domain is also needed for polarized budding (Lodge *et al*., 1994; Lodge *et al*., 1997), which indicates that an intracellular interaction between MA and TM is required. During early stages of HIV replication, a fraction of MA remains associated with the viral preintegration complex (Bukrinsky *et al*., 1993a; Miller

et al., 1997). HIV can productively infect non-dividing cells because the viral genome/PIC is actively transported through the nucleopore (Bukrinsky *et al*., 1992; Lewis *et al*., 1992; Weinberg *et al*., 1991) and MA is one of the viral components which confer nucleophilic properties to PIC.

A putative nuclear localization signal in the N-terminus of MA has been identified (Bukrinsky *et al*., 1993b; Gallay *et al*., 1995), which maps to the exposed basic patch on the globular head that has been implicated in Gag membrane binding (Bukrinsky *et al*., 1993a; Zhou *et al*., 1994). It has been reported that HIV-1 mutants that carry substitutions in the MA NLS replicate efficiently in dividing cells but not in growth arrested cells or terminally differentiated macrophages, which is consistent with a role of MA in nuclear import of the PIC (Bukrinsky *et al*., 1993b; Heinzinger *et al*., 1994; von Schwedler *et al*., 1994). Additionally a nuclear export activity has been described for MA that counteracts the NLS during virus production, thus ensuring that Gag is available for virus assembly (Dupont *et al*., 1999). Recently MA has also been shown to play a role in the early steps in the particle assembly by interacting directly with δ subunit of the AP-3 complex and disruption of this interaction prevents Gag from reaching the multi vesicular body compartment and inhibits particle formation (Dong *et al*., 2005). MA was also found to interact with translational elongation factor 1-alpha (EF1-α) and inhibiting translation, which may serve to release the viral RNA from polysomes, allowing the RNA to be packed into nascent virions (Cimarelli and Luban, 1999).

2.3.2.3 Capsid.

Capsid (CA) has crucial roles in particle assembly and also after entering into a new target cell. In the mature virion, CA forms the shell of the core, which is occasionally tubular but most often conical, a feature that distinguishes lentiviruses such as HIV-1 from most other retroviruses. CA has

two predominantly α-helical domains that are connected through a flexible linker region (Gamble *et al*., 1997; Gitti *et al*., 1996; Momany *et al*., 1996). Both domains have different roles in virus morphogenesis. The N-terminal domain, which comprises two thirds of HIV-1 CA, is required for the formation of the mature core but is dispensable for the assembly of immature virus particles (Borsetti *et al*., 1998; Dorfman *et al*., 1994a; Reicin *et al*., 1996; Reicin *et al*., 1995; Wang and Barklis 1993). In contrast, the C-terminal CA domain is crucial both for particle assembly and for core formation (Dorfman *et al*., 1994a; Mammano *et al*., 1994; McDermott *et al*., 1996; Reicin *et al*., 1995). The N-terminal domain of HIV-1 CA interacts with human peptidyl-prolyl *cis-trans* isomerase, cyclophilin A (CypA) leading to specific incorporation of this ubiquitous cytosolic host protein into virions (Franke *et al*., 1994b; Luban *et al*., 1993; Thali *et al*., 1994). Cyclophilins catalyze the isomerization of peptidyl-prolyl bonds, a rate limiting step in protein folding. They function as chaperones, having a broad sub-cellular distribution, lowered levels of virion associated cyclophilin A results in lowered levels of virion infectivity (Thali *et al*., 1994). The ratio of CA: CypA in the virion is about 10:1 and the interaction between CA and CypA has been shown to be major determinant in species specific restriction of HIV to many non-human primate cells.

2.3.2.4 Nucleocapsid.

The nucleocapsid (NC) domain which lies C-terminal to CA, harbors two copies of CCHC-type zinc finger motif that is present at least once in the NC proteins of all retroviruses. Each of the two conserved CCHC motifs in HIV NC coordinates a zinc ion (Bess *et al*., 1992; Summers *et al*., 1992; Summers *et al*., 1990), and both motifs are absolutely essential for viral replication (Aldovini and young, 1990; Dorfman *et al*., 1993; Gorelick *et al*., 1993; Gorelick *et al*., 1990). The NC domain is essential for the specific packaging of the two copies of genomic viral RNA into assembling particles (Berkowitz *et al*., 1996). Studies involving point mutations and deletions in NC have shown that NC

also plays a role in virus particle assembly (Dorfman *et al*., 1993; Dawson and Yu, 1998; Zhang and Barklis, 1997). NC plays a central role in Gag multimerization which is required for assembly of viral particles of normal density (Burniston *et al*., 1999; Franke *et al*., 1994a; Bowzard *et al*., 1998). Recently, NC has been shown to interact directly with cellular cytidine deaminase APOBEC3G and thereby incorporating it into virus particles. (Luo *et al*., 2004).

2.3.2.5 p6.

While MA, CA, NC are common to all retroviruses, the p6 domain is present at the C-terminus of the Gag polyprotein only in HIV/SIV and other lentiviruses. The p6 domain is essential for HIV propagation in cell culture, and small deletions in HIV p6 have been associated with non progressive infection in humans (Gottlinger *et al*., 1991; Alexander *et al*., 2000). A major function of p6 is to promote the detachment of assembled virions from the cell surface. Within p6 the major determinant for virus detachment function is the conserved P(T/S)APP motif near the N-terminus (Gottlinger *et al*., 1991; Huang *et al*., 1995). Functionally equivalent regions have also been found in Gag polyproteins of other retroviruses (Parent *et al*., 1995; Wills *et al*., 1994; Xiang *et al*., 1996; Yasuda and Hunter, 1998; Yuan *et al*., 1999). These regions are now commonly called late assembly domains (L) (Garnier *et al*., 1998a). A small fraction of p6 in mature HIV virions is monoubiquitinated, the role of ubiquitylation in the L domain was implied by the finding that inhibitors of the proteasome interfere with the budding of retroviruses and rhabdoviruses at a late stage, similar to what is seen in the absence of a late domain (Harty *et al*., 2001; Patnaik *et al*., 2000; Schubert *et al*., 2000; Strack *et al*., 2000). P6 was also shown to interact with Tsg101, a product of tumor susceptibility gene *tsg* 101 which functions in the late endosomal trafficking (Garrus *et al*., 2001; Verplank *et al*., 2001). The binding of Tsg101 to HIV p6 depends on the P(T/S)APP motif and is enhanced if p6 is modified by ubiquitin (Garrus *et*

al.,2001; Verplank *et al*., 2001). Furthermore HIV budding is arrested at a late stage if cellular Tsg101 is depleted, and is restored if Tsg101 expression is restored (Garrus *et al*., 2001).

2.3.3. Viral enzymes.

Downstream of *gag* lies the most conserved region of the lentivirus genome, the *pol* gene, which encodes three viral enzymes, the protease (PR), reverse transcriptase (RT) and integrase (IN). The RT and IN are required for reverse transcription of the viral genome and for the integration of the viral DNA into the host genome, respectively. The PR protein plays a critical role in the life cycle by mediating the production of mature infectious virions. The *pol* gene products are derived by the enzymatic cleavage of 160 kDa Gag-Pol fusion protein referred to as Pr160 Gag-Pol. The Pr 160 Gag-Pol is generated by a ribosomal frame shift event, which is triggered by a specific *cis*-acting heptanucleotide RNA motif followed by a short stem loop in the distal region of *gag* RNA. When ribosomes encounter this motif, approximately 5-10% of the translational events are shifted to the *pol* reading frame without interrupting translation (Crawford and Goff, 1985). This frequency of ribosomal frame shift explains why the Gag and the Gag-pol precursors are produced at the ratio of approximately 20:1.

2.3.3.1 Protease.

The mature form of protease (PR) is 99 amino acids in length with a molecular mass of 10 kDa (Katz and Skalka, 1994). The retroviral PR proteins are related to cellular aspartyl proteases because the sequence Asp-Thr/Ser-Gly is conserved in the active site of both the viral and cellular enzymes. The first cleavage events catalyzed by the retroviral PRs during or immediately after the virion release from the cell serves to liberate PR from the Gag-Pol precursor (Crawford and Goff, 1985). It is still unclear whether this initial cleavage event takes place in *cis* or *trans*. In either case following the release

of PR from the Gag-Pol precursor, the dimeric enzyme cleaves a number of sites in both Gag and Gag-Pol. The efficiency of the cleavage depends on two factors, the amino acid sequence at the site of cleavage and the degree of exposure of the cleavage site (Crawford and Goff, 1985). The PR protein appears to be most active during the budding process just prior to the release of the virus particle from the cell, leading to virus maturation and formation of a tightly packed cone shaped core. During viral maturation the PR protein cleaves the Pol polypeptide away from Gag and further digests it to separate itself (p10), RT (p51), RNaseH (p15) and Integrase (p32). These cleavages do not occur efficiently for example, roughly 50% of the RT protein remains linked to RNaseH as a single polypeptide (p66) (Vogt, 1996). The HIV-1 PR activity is not restricted to cleavage of Gag or Gag-Pol precursors. The Nef protein is also a substrate for PR cleavage, although the implications of Nef cleavage are unclear (Miller *et al*., 1997). In addition to cleavage of viral proteins the protease has been reported to act on a number of cellular proteins as well. The role of such events in virus replication and virus induced cytotoxicity has not been fully elucidated. The three dimensional structure of protease dimer has led to develop a class of drugs directed towards inhibiting the HIV protease function (Wlodawer and Erickson, 1993). These antiviral compounds (Indinavir, Saquinavir) together with RT inhibitors (AZT and 3TC) are used for highly active antiretroviral therapy or HAART, and have greatly improved the treatment regimen for HIV infected individuals.

2.3.3.2 Reverse transcriptase.

Retroviruses possess the ability to convert their single-stranded RNA genome into double-stranded DNA during the early stages of the viral replication cycle. This reaction is catalyzed by the viral RT along with its RNaseH activity. Retroviral RTs have three enzymatic activities 1) RNA – dependent DNA polymerase for minus strand DNA synthesis, 2) RNaseH for degradation of tRNA primer and genomic RNA in the DNA-RNA hybrid

intermediate (Hostomsky *et al*., 1991) and 3) DNA dependent DNA polymerase for the second or plus–strand DNA synthesis (Baltimore, 1970; Temin and Mizutani, 1970). Genetic analyses and site-directed mutagenesis have demonstrated that RT and RNaseH are indispensable for viral replication. Both enzymes are processed from Pr160 Gag-pol precursor protein in two steps by the PR protein during the virion assembly. The p66 protein containing RT and RNaseH is first cleaved from this polyprotein to form a homodimer. Subsequently one subunit of p66 in this homodimer is cleaved by PR near the C-terminus to yield a heterodimer composed of p51 and p66. Both heterodimer and the p66 homodimer display RT and RNaseH activities whereas the p51 subunit alone is not active. The RT activity is routinely used in the laboratory to quantitatively monitor the levels of virus present in the culture supernatant as a measure of *in vitro* viral replication.

The high rate of variation among HIV is largely a consequence of error prone nature of RT, which lacks an exonucleotide proof-reading activity, and the frequent template switching during reverse transcription. The *in vivo* mutation rate of HIV is due to substitutions, frame shifts, simple deletions and deletions with insertions, and has been estimated to be $3x10^{-5}$ per cycle of replication (Mansky, 1998). The HIV RT enzyme has long been a target for antiviral compounds. A number of RT inhibitors have been developed, including nucleoside analogs such as 3'-azido-3'deoxythymidine (AZT or Zidovudine), 2',3'-dideoxyionosine(ddI or didanosine) and 2'3'-dideoxycytidine (ddC or Zalcitabine) and the non-nucleoside inhibitors such as nevirapine and delavirdine. The nucleoside analogs act as chain terminators, whereas the non-nucleoside compounds inhibit DNA polymerization by binding to a small hydrophobic pocket near the RT active site inducing an allosteric change at the active site. Unfortunately, resistance to these compounds develops rapidly in patients, perhaps reflecting the fact that resistant variants exist even before the initiation of the therapy (Coffin, 1995).

2.3.3.3 Integrase.

One hallmark of retroviral replication is integration of DNA copy of the viral RNA genome into the host chromosome following reverse transcription. The integrated viral DNA (provirus) serves as a template for the synthesis of viral RNAs and is maintained as a part of the host genome for life time of the infected cell. This integration of the viral genome into the host genome is catalyzed by the viral Integrase. The C-terminus of the HIV and SIV Gag-Pol precursor is proteolytically cleaved by PR to produce the 32 kDa Integrase protein (IN). Genetic studies of the viral mutants have shown that this enzyme is required for replication in T-cell lines as well as in primary lymphocytes and macrophages. During integration, IN protein removes two nucleotides from the 3' termini of both strands of full length, linear viral DNA, generating a preintegration substrate with 3' recessed ends. This 3' end processing reaction can take place in the cytoplasm of the infected cell. Then in the nucleus IN catalyzes a staggered cleavage of cellular targets. Selection of the host DNA target site by IN is essentially sequence independent. The 3' recessed ends of the viral DNA are joined to the 5' overhanging termini of the cleaved cellular DNA in the strand transfer step. The cellular machinery perhaps then in conjunction with IN, fills the gaps by completing the integration process. The integrated HIV provirus is flanked by a 5 base pair direct repeat and terminates with the di-nucleotides 5' TG and CA 3'.

The direct repeat results from the duplication of the cellular target sequences (Fujiwara and Mizuuchi, 1988). No energy source like ATP is required for this integration reaction but divalent metal ions such as Mg++ or Mn++ are required (Crigie *et al*., 1990; Katz *et al*., 1990). The accessibility of the chromosomal DNA within chromatin, rather than specific DNA sequences, seems to influence the choice of integration sites. Kinks in the DNA within

chromatin are thus hotspots for integration, at least *in vitro*. Preferential integration into regions of open and transcriptionally active chromatin may facilitate expression of provirus. Viral genes are not efficiently expressed from non-integrated proviral DNA (Bushman, 1994; Pruss *et al*., 1994; Wiskerchen and Muesing, 1995). Retroviral IN proteins are composed of three structurally and functionally distinct domains that include an N-terminal zinc finger domain, a core domain and a relatively non-conserved C-terminal domain.

2.3.4 The Envelope glycoprotein.

The Env glycoprotein molecules on the surface of HIV and SIV particles bind to CD4 receptors located on the plasma membrane of CD4 T lymphocytes, monocytes, macrophages and dendritic cells. Apart from CD4, for efficient membrane fusion and infection HIV/SIV requires additional chemokine co-receptors called CXCR4 and CCR5. T-cell tropic viruses utilize CXCR4 where as macrophage tropic viruses use CCR5. In recognition of the importance of a co-receptor in determining HIV-tropism, a nomenclature scheme was devised based on co-receptor usage. Macrophage tropic isolates that utilized CCR5 were designated R5 isolates, while T-cell tropic isolates preferentially used CXCR4 are called X4 viruses. Dual tropic isolates that utilized both CCR5 and CXCR4 were designated as R5X4 viruses (Berger *et al*., 1998). Following the attachment step, the Env glycoprotein mediates uptake of virions into cells by fusion of viral and cellular membranes. In addition, the viral glycoprotein is also responsible for induction of syncytia in tissue culture cells and is the major target for antiviral immune responses in the infected host.

The HIV Env glycoprotein is synthesized from a singly spliced 4.3 kb *vpu*/*env* bicistronic mRNA and the translation occurs on ribosomes associated with the rough endoplasmic reticulum. The 160 kDa polyprotein precursor (gp160) is an 850 to 880 amino acid integral membrane protein that is

anchored to cell membranes by a hydrophobic stop-transfer signal in the domain destined to be the mature transmembrane envelope glycoprotein gp 41. The gp160 is co-translationally glycosylated in the ER, and rapidly associates with host chaperone Bip/GRP78, by disulphide bonds and oligomerizes (Earl *et al*., 1991; Willey *et al*., 1988). It can oligomerize as dimers, trimers and tetramers but the predominant form is a tetramer. Further gp160 is transported to the golgi and proteolytically cleaved by cellular enzymes into mature surface glycoprotein gp120 of about 550 amino acids, and the transmembrane glycoprotein gp41 of about 350 amino acids. The cellular enzyme responsible for cleavage of retroviral Env precursors uses a highly conserved Lys/Arg-XLys/Arg-Arg motif and is a furin or a furin-like protease. The surface glycoprotein, gp120 is a highly glycosylated hydrophilic protein positioned on the external surface of virion as well as plasma membranes of infected cells. This protein has a binding domain for the CD4 receptor (Landau *et al*., 1988). The transmembrane glycoprotein, gp41, is relatively hydrophobic and traverses the lipid bilayer membranes of virions or infected cells. Thus, gp41 is classified as a type 1 integral membrane protein. In its mature form, the Env glycoprotein is a heterodimer consisting of gp120 and gp41 subunits held together by non-covalent interactions involving several points of contacts between the two subunits. Mutagenesis studies have demonstrated that amino acid residues in both the N-and C-termini of gp120 are critical for maintaining its association with gp41 (Freed *et al*., 1989; McCune *et al*., 1988). The precise structural features in the gp41 subunit contacting the gp120 subunit have not been defined, although it has been proposed that a disulphide loop in gp41 may form a knob that fits into a pocket in the folded gp120 subunit.

The amino acid sequence analysis of HIV-1 gp120 reveals 24 potential sites for N-linked glycosylation (Asn-X-Ser/Thr) with about 13 of these motifs being conserved in different viral isolates. Because of this extensive

glycosylation, computer derived models suggest that very few regions of the gp120 peptide backbone protrude from the carbohydrate mass (Nara *et al*., 1991). Further, the sequence of HIV-1 gp120 shows 18 cysteine residues. Because the cysteine residues are highly conserved in the glycoproteins of diverse HIV-1 and HIV2/SIV strains, disulphide bonds are presumed to be critical for the structure and function of these viral proteins (Hoxie, 1991). A model for the gp120 subunit, based partly on biochemical analysis, shows nine intra-chain disulphide bonds. This disulphide bonding pattern delineates gp120 into several functional regions, which include a conformation-dependent domain for recognition of the CD4 receptor (Leonard *et al*., 1990).

The gp41 sequence contains four potential glycosylation sites and three cysteine residues (Freed *et al*., 1990). About 20 amino acids at the N-terminus of gp41 are hydrophobic and form the fusion peptide (amino acids 512 to 527 of gp160) that is required for fusion of the virion membrane with the plasma membrane of the cell during the entry step in viral replication (Horth *et al*., 1991; Kowalski *et al*., 1987). A second hydrophobic domain spans virions and cell membranes and thereby enables gp41 to serve as an anchor for the Env glycoprotein heterodimer. The region between the two hydrophobic domains contains an external highly conserved sequence (leu 553 to leu 590) of gp160, similar to the leucine zipper motif and is required for protein-protein interactions. Mutation in this leucine motif of gp41 blocks viral infectivity and cell fusion without affecting synthesis, oligomer formation, transport, and proteolytic processing of the Env glycoprotein (Cao *et al*., 1993; Chen *et al*., 1993b; Dubay *et al*., 1992). During viral entry, a portion of the gp41 ectodomain (including leucine zipper) has been proposed to adopt a coiled coil conformation, which facilitates insertion of the gp41 fusion peptide into the target cell membrane (Wild *et al*., 1994).

2.3.5 Regulatory proteins.

2.3.5.1 Tat.

The Tat protein is a potent transcriptional activator of the HIV long terminal repeat (LTR) promoter element and is essential for replication in all cell types. Tat binds to a RNA stem loop structure called Tat activation region (TAR) located immediately 3' to the LTR transcription start site. Tat has two functional domains such as N-terminal co-factor binding domain, and an arginine-rich RNA binding motif (ARM), extending from amino acids 49 to 58 which acts as a nuclear localization signal (Hauber *et al*., 1989; Chin *et al*., 1991; Ruben *et al*., 1989; Dang and Lee, 1989). In the absence of Tat, most of the initiated transcripts from HIV LTR are terminated prematurely within ~200 nucleotides from the transcription start site (Kao *et al*., 1987). In association with cyclin T1, Tat binds to TAR and recruits the cellular-cyclin dependent kinase 9 (Cdk9) to the HIV LTR (Wei *et al*., 1998). In the positive transcription elongation factor b (P-TEFb) complex, Cdk9 phosphorylates the C-terminal domain of RNA pol II, which marks the transition from initiation to elongation of eukaryotic transcription (Fig. 5).

The ARM motif in Tat binds to the 52 nucleotide bulge region in TAR and a shorter ARM in cyclin T1 which is called Tat-TAR recognition motif (TRM).This forms a high affinity RNA binding unit that is required for Tat transactivation. Tat also recruits mammalian capping enzyme (Mce1) and stimulates the guanyltransferase and triphosphatase activity of Mce1 thereby enhancing the low efficiency cap formation on TAR stem-loop RNA (Chiu *et al*., 2001). Chromatin is also an important regulatory component of HIV-1 transcription. Tat has been shown to associate with histone acetyl transferases (HATs) p300/CBP, p300/CBP associating factor (PCAF) and hGCN5 (Benkirane *et al*., 1998; Hottiger and Nabel, 1998; Marzio *et al*., 1998).

Tat recruited HATs presumably acetylate histones in LTR proximal nucleosomes to potentiate transcription.

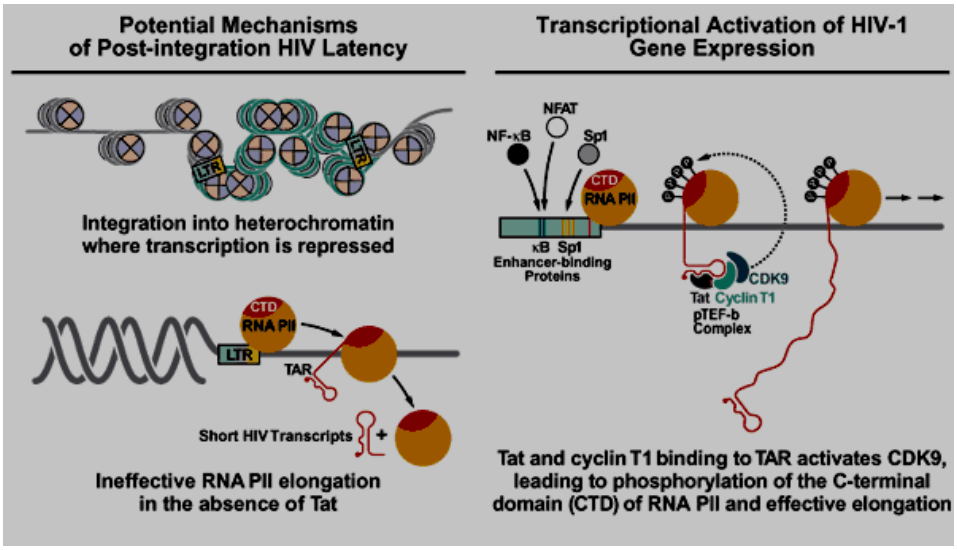


Figure 5: Mechanism of Tat mediated transcriptional activation.

2.3.5.2 Rev.

Rev is a 116 amino acid protein encoded by fully spliced HIV mRNA and is one of the early gene products synthesized along with Tat and Nef. Eukaryotic cells have evolved mechanisms to retain incompletely spliced RNAs in the nucleus until the splicing is complete. Though HIV utilizes cellular machinery for splicing, it overcomes the hurdle to transport incompletely spliced transcripts which include the complete viral genome and transcripts for other viral proteins by Rev. Rev is a RNA export factor that is able to induce the efficient nuclear export and hence expression of incompletely spliced viral transcripts (Malim *et al*., 1989). Rev contains an arginine-rich motif in the N-terminus that serves as a nuclear localization signal by which it binds directly to nuclear transport receptor importin-β for nuclear import (Henderson and Percipalle, 1997). Rev binds to a RNA stem loop structure called the rev

response element (RRE) within the HIV *env* gene (Malim *et al*., 1989) in all spliced and unspliced viral transcripts and shifts the balance from multiply spliced transcripts encoding Tat, Rev, and Nef in the early stages of replication to singly spliced and unspliced transcripts encoding the viral structural proteins in the late stages of replication (Cullen, 1991). Rev also has a 10 amino acid leucine-rich sequence that serves as a nuclear export signal (NES). Mutation in any of the Leucine residues at position 78,81and 83 within this domain eliminates Rev export activity. Rev is exported by a nuclear export receptor called Crm1/exportin-1 that is highly conserved in yeast and humans (Fornerod *et al*., 1997; Neville *et al*., 1997; Stade *et al*., 1997).

2.3.6 Accessory proteins.

In addition to the *gag*, *pol*, and *env* genes that are present in all retroviruses and the *tat* and *rev* regulatory genes, HIV-1 contains four additional genes: *nef*, *vif*, *vpr* and *vpu*. HIV-2/SIVsm does not contain *vpu*, but instead harbors another gene, *vpx*. The accessory proteins are not absolutely required for viral replication in all *in vitro* systems, but represent critical virulence factors *in vivo*.

2.3.6.1 Vif.

Vif (virion infectivity factor) encodes an approximately 220 amino acid cytoplasmic protein that is expressed from a multiply spliced, Rev-dependent mRNA (Garret *et al*., 1991). The Vif protein, which modulates viral infectivity (Dettenhofer *et al*., 2000; Gabuzda *et al*., 1992; Gibbs *et al*., 1994;) and pathogenecity (Desrosiers *et al*., 1998; Harmache *et al*., 1996; Inoshima *et al*., 1996; Inoshima *et al*., 1998) is present in nearly all lentiviruses, excluding equine infectious anemia virus. Cell types like primary T cells and transformed T cells lines such as PM1, HUT78 where *vif* is required for efficient HIV replication are termed nonpermissive (Gabuzda *et al*., 1992; von Schwedler *et al*., 1993) and cell types like SupT1, CEMss, C8166 where *vif* is not required

are termed permissive. Differential expression screening of non-permissive cells over permissive cells lead to identification of a protein which was first named CEM15, that specifically inhibited the replication of *vif* – minus HIV-1 (Sheehy *et al*., 2002). The CEM15 is now known as APOBEC3G a cytidine deaminase. The main function of Vif has now shown to block the antiviral activity of APOBEC3G. Vif mutant but not wild type, HIV produced in the presence of APOBEC3G undergoes hyper mutations in the newly synthesized viral DNA due to C-to-U modification during minus-strand viral DNA synthesis. Following deamination by APOBEC3G, the heavily modified viral DNA is recognized by the host uracil-DNA glycosidase, which removes dU residues in DNA. The resulting DNA is in turn recognized and degraded by the DNA base excision repair enzymes. The viral cDNA is highly sensitive to this mechanism of attack because the transient DNA intermediates of reverse transcription are single stranded following RNaseH action and can not be repaired like double stranded DNA, using the complementary strand. Alternatively, if reverse transcription is successfully completed at low efficiency and the resulting double strand is integrated into the host genome, the C to U conversion on the minus strand leads to corresponding G to A hyper mutation on the plus strand. The resulting provirus is so heavily mutated that it may not be able to synthesize functional viral proteins (Harris *et al*., 2003; Lecossier *et al*., 2003; Mangeat *et al*., 2004; Zhang *et al*., 2003). HIV-1 Vif also induces polyubiquitylation of human APOBEC3G leading to proteasomal mediated degradation (Conticello *et al*., 2003; Marin *et al*., 2003; Mehle *et al*., 2003).

2.3.6.2 Vpr.

The Vpr protein is an accessory protein found both in HIV and SIV and is a virion associated protein that is packaged into the virus particles in molar amounts equivalent to those of the Gag protein (Lu *et al*., 1993). Vpr displays several distinct activities in host cells, these include nuclear import of PIC into the nucleus of non-dividing cells like primary macrophages (Heinzinger *et al*.,

1994), induction of G2/M phase arrest during cell cycle (He *et al*., 1995) and apoptosis (Stewart *et al*., 1997). These functions were shown to be functionally independent of each other (Chen *et al*., 1999; Elder *et al*., 2000) and have been demonstrated in a wide variety of eukaryotic cells ranging from humans to yeast indicating that Vpr most likely affects highly conserved cellular processes. All regulatory and accessory proteins are targeted by HIV-1 specific CD8 CTLs (Addo *et al*., 2002), however Vpr is preferentially targeted by the CD8 T lymphocytes during the acute phase of the viral infection (Altfeld *et al*., 2001; Mothe *et al*., 2002). Vpr suppresses antigen specific CD8 mediated CTL and immune responses (Ayyavoo *et al*., 2002) by inhibiting T cell clonal expansion through suppression of T cell proliferation and inducing cell cycle arrest at G2/M transition (Poon *et al*., 1998). Vpr also inhibits host inflammatory responses by down regulating pro-inflammatory cytokines (TNF α and IL-12) and chemokines (RANTES, MIP1α and MIP1 β) in a manner similar to that of glucocorticoids (Refaeli *et al*., 1995; Ayyavoo *et al*., 1997).

Vpr induced cell cycle G2/M arrest maps to a 26 amino acid carboxy-terminal basic domain. Cells expressing Vpr contain very low levels of p34cdc2-cyclin B kinase activity, although both the proteins are expressed (Re *et al*., 1995). The activity of p34cdc2-cyclin B kinase is critical for the transition from G2 to M phase and this requires the removal of phosphate residues on p34cdc2 by the phosphatase cdc25C. Vpr inhibits cdc25C phosphatase activity (Elder *et al*., 2001; Bartz *et al*., 1996) and activates wee1 kinase (Elder *et al*., 2001; Yuan *et al*., 2004) to promote phosphorylation of Cdc2/Cdk1 during the induction of G2 arrest. An interesting rationale for the Vpr mediated arrest of cells in G2 is provided by the observation that the HIV-1 LTR promoter is more active in G2 arrested cells (Goh *et al*., 1998).

Vpr also plays a major role in transport of viral genome into the nucleus of non-dividing cells. The HIV-1 Vpr NLS, which extends over essentially the

entire amino-terminal 70 amino acids is a non-canonical NLS i.e., distinct from the basic NLS prototype. Fusion of heterologous protein like β-Gal to the Vpr NLS induces not only nuclear import, but also a marked accumulation of the fusion protein at nuclear pores (Vodicka *et al*., 1998). Vpr can directly interact with nucleoporins thus performing an activity attributed to importin-β (Vodicka *et al*., 1998; Fouchier *et al*., 1998; Le Rouzic *et al*., 2002). Vpr can also bind to importin-α but the binding site is different from that of classical NLS (Popov *et al*., 1998). The principal impact of HIV on immune system is destruction of CD4 T lymphocytes by apoptosis and Vpr plays a major role. Vpr induces apoptosis in infected cells by activating the intrinsic pathway of apoptosis. This pathway is activated by the release of cytochrome C from the inner membrane space of mitochondria into the cytoplasm (Green and Kroemer, 2004).

2.3.6.3 Vpu.

Vpu is present only in HIV-1 and closely related SIVcpz and SIVgsn isolates (Cohen *et al*., 1988; Huet *et al*., 1990; Strebel *et al*., 1988). Vpu is an oligomeric type 1 integral membrane protein (Maldarelli *et al*., 1991) consisting of an amino terminal transmembrane domain and a carboxy-terminal cytoplasmic tail. Vpu induces degradation of CD4 molecules trapped in intracellular complexes with Env, thus allowing gp160 to resume transport towards cell surface (Bour *et al*., 1995a; Crise *et al*., 1990; Jabbar and Nayak, 1990). Simultaneous synthesis of both HIV-1 envelope and CD4 in a single cell results in the formation of Env-CD4 complexes in the ER that are retained and eventually degraded. Vpu directly interacts with CD4 through a domain extending from residues 416 to 418 (EKTT) in the CD4 cytoplasmic domain while it is localized in the ER to target CD4 to an ER associated degradation pathway (Willey *et al*., 1992). The two conserved serine residues at positions 52 and 56 in the cytoplasmic domain of Vpu are critical for CD4 degradation (Lenburg and Landau, 1993; Vincent *et al*., 1993; Schubert and Strebel, 1994). These residues interact with human β-transducin repeat containing

protein (h-βTrCP) (Margottin *et al*., 1998), which consists of a series of WD repeat elements required for binding to Vpu, and contains an F-box domain that functions as a connector between target proteins and the ubiquitin-dependent-proteasome machinery (Bai *et al*., 1996; Schubert *et al*., 1998). Vpu also aids in the enhancement of virion release from infected cells and this function is dependent on hydrophobic amino terminal transmembrane domain. Vpu facilitates virion release by promoting the budding of virions from plasma membrane (Klimkait *et al*., 1990).

2.3.6.4 Nef.

Nef (negative factor) is conserved in all primate lentiviruses and is expressed in abundance during the early phase of HIV infection (Guy *et al*., 1987; Klotman *et al*., 1991). The *nef* gene is critical for pathogenesis and development of AIDS in humans as well as in simians. Long term survivors of HIV infection are commonly associated with a deletion in *nef* gene or have defective *nef* alleles (Deacon *et al*., 1995; Mariani *et al*., 1996). Nef protein from HIV and SIV has been shown to down regulate the steady state levels of CD4 on the cell surface (Aiken *et al*., 1994; Piguet *et al*., 1999; Greenway *et al*., 2003). Down regulation of CD4 leads to enhancement of viral particle release by preventing the sequestration of viral envelope by the CD4 receptor (Lama, 2003). Down regulation also prevents super infection, an event that leads to premature death of the infected host cell (Lama, 2003). Nef down modulates MHC-1 and this down regulation protects HIV infected cells from CTL killing and provides a selective advantage for viral persistence and replication *in vivo* (Collins *et al*., 1998). Nef selectively down regulates HLA-A and HLA-B which present antigens to CTLs, but not HLA-C and HLA-E which helps to protect cells from lysis by natural killer cells (Garcia and Foster, 1996; Swigut *et al*., 2000; Cohen *et al*., 1999). Nef also disrupts MHC-II antigen presentation by down regulating the surface expression of mature MHC-II.

Nef induces the expression of both Fas (CD95) and Fas ligand (CD95L) in HIV infected cells (Xu *et al*., 1999; Zauli *et al* 1999), with CD95L aiding immune evasion by inducing the apoptosis of HIV specific CTLs (Xu *et al*., 1999). The apoptosis signal regulating kinase (ASK1) is a key signaling intermediate in the Fas and TNFα death signaling pathways and has been reported to bind to Nef (Geleziunas *et al*., 2001). Nef also suppresses death signaling by Bad, a proapoptotic member of the Bcl-2 protein family, whose expression is induced by HIV and that triggers apoptosis at the level of mitochondria (Gross *et al*., 1999). Nef also interacts with p53 tumor suppressor protein and this interaction results in the destabilization of p53, thereby decreasing its pro-apoptotic, transcriptional, and DNA binding activities thereby protecting the HIV infected cells from p53 mediated apoptosis (Greenway *et al*., 2002).

2.3.6.5 Vpx.

Human immunodeficiency virus type-2 (HIV-2) and Simian immunodeficiency virus from sooty mangabeys (SIVsm) belong to a closely related group of lentiviruses (Chakrabarti *et al*, 1987; Dietrich *et al*., 1989; Guyader *et al*., 1987; Hirsch *et al*., 1989). Unlike other lentiviruses these viruses contain *vpx* gene in addition to a *vpr* gene that is present in HIV-1. This gene was initially designated as X orf and is found only in these viruses (Franchini *et al*., 1988; Henderson *et al*., 1988; Kappes *et al*., 1988; Yu *et al*., 1988), and is absent in other lentiviruses like HIV-1 and SIVcpz.

Striking amino acid sequence homology between HIV-2/SIVsm Vpr and Vpx suggests that they arose by gene duplication and thus may share a common ancestor (Tristem *et al*., 1990; Tristem *et al*., 1992). Even though HIV-2/SIVsm Vpr and Vpx share considerable sequence similarity, they have distinct and non-redundant functions. Vpr induces cell cycle arrest at G2/M phase (Fletcher *et al*., 1996; Rogel *et al*., 1995), whereas Vpx was found to be

mainly involved in the nuclear import of preintegration complex (PIC) (Fletcher *et al*., 1996).

Vpx is dispensable for viral replication in established human T lymphocyte cell lines (Guyader *et al*., 1989; Hu *et al*., 1989; Kappes *et al*., 1991; Marcon *et al*., 1991; Mahalingam *et al*., 2001), whereas it is essential for replication in macrophages (Yu *et al*., 1991; Mahalingam *et al*., 2001). Vpx is also essential for efficient *in vivo* dissemination and spread of SIVsm following mucosal and intravenous infection of macaques (Hirsch *et al*., 1998). Within viral particles Vpx seems to be localized within viral core (Kewalramani and Emerman, 1996). Vpx is efficiently packaged in the progeny virions in molar amounts equivalent to that of Gag by its interaction with C-terminus of Gag (Henderson *et al*., 1993; Pancio and Ratner, 1998; Accola *et al*., 1999; Kappes *et al*., 1993). Additionally HIV-2 Vpx appears to co-localize with HIV-2 Gag at the inner surface of the plasma membrane of the infected cells (Kappes *et al*., 1993).Though Vpx was shown to play major role in PIC nuclear import and replication in non-dividing target cells but the signals and pathways for Vpx nuclear transport and its role in PIC transport remain unknown.

2.4 HIV life cycle

The process of viral entry involves fusion of the viral envelope with the host cell membrane and requires the specific interaction of the viral envelope with specific cell surface receptors. The viral envelope proteins, gp120 and gp41, form a trimeric functional unit consisting of three molecules of gp120 exposed on the virion surface and three molecules of gp41 inserted into the viral lipid membrane. Trimeric gp120 on the surface of the virion binds CD4 on the surface of the target cell, inducing a conformational change in the envelope proteins that in turn allows binding of the virion to a specific subset of chemokine receptors on the cell surface (Kwong *et al*., 1998). Twelve chemokine receptors can function as HIV coreceptors in cultured cells, but

only two are known to play a role *in vivo* (Doms and Trono, 2000). One of these, CCR5, binds macrophage-tropic, non-syncytium-inducing (R5) viruses, which are associated with mucosal and intravenous transmission of HIV infection. The other, CXCR4, binds T-cell-tropic, syncytium-inducing (X4) viruses, which are frequently found during the later stages of disease (Scarlatti *et al*., 1997). The binding of gp120 to CD4 and the chemokine co-receptors produces an additional radical conformational change in gp41 (Chan and Kim, 1998). Assembled as a trimer on the virion membrane, this coiled-coil protein springs open, projecting three peptide fusion domains that “harpoon" the lipid bilayer of the target cell. The fusion domains then form hairpin-like structures that draw the virion and cell membranes together to promote fusion, leading to the release of the viral core into the cell interior.

2.4.1 Cytoplasmic events

Once inside the cell, the virion undergoes uncoating while it is still associated with the plasma membrane (Fig. 6). This poorly understood process involves phosphorylation of viral matrix proteins by a mitogen-activated protein (MAP) kinase (Cartier *et al*.,1999) and additional actions of cyclophilin A (Franke *et al*., 1994) along with viral proteins Nef (Schaeffer *et al*., 2001) and Vif (Ohagen and Gabuzda, 2000). After the virion is uncoated, the viral reverse transcription complex is released from the plasma membrane (Karageorgos *et al*., 1993) and docks with actin microfilaments (Bukrinskaya *et al*., 1998). This interaction, mediated by the phosphorylated MA promotes efficient viral DNA synthesis. Reverse transcription yields the preintegration complex (PIC) (Fig.7) which is composed of double-stranded viral cDNA, Integrase, MA, Vpr, Reverse transcriptase, and the high mobility group DNA-binding cellular protein HMGI(Y) (Miller *et al*., 1997). In addition to these proteins, PIC in HIV-2/SIVsm lineage viruses contains Vpx also. The PIC move towards the nucleus by using microtubules as a conduit (McDonald *et al*., 2002).

Unlike most animal retroviruses, HIV can infect non-dividing cells, such as terminally differentiated macrophages (Weinberg *et al*., 1991) and this requires an ability to cross the intact nuclear membrane. Three different proteins Integrase (Gallay *et al*., 1997) MA (Bukrinsky *et al*., 1993) and Vpr (Heinzinger *et al*., 1994) have been implicated in the nuclear import of HIV-1 PIC. Additionally a triple helical DNA domain or 'DNA flap' which forms as a result of discontinuous plus-strand synthesis during reverse transcription may also help in nuclear import of PIC by binding a host protein containing a nuclear targeting signal (Zennou *et al*., 2000). However, a recent publication questions the contributions of the nuclear import signal in Integrase and the

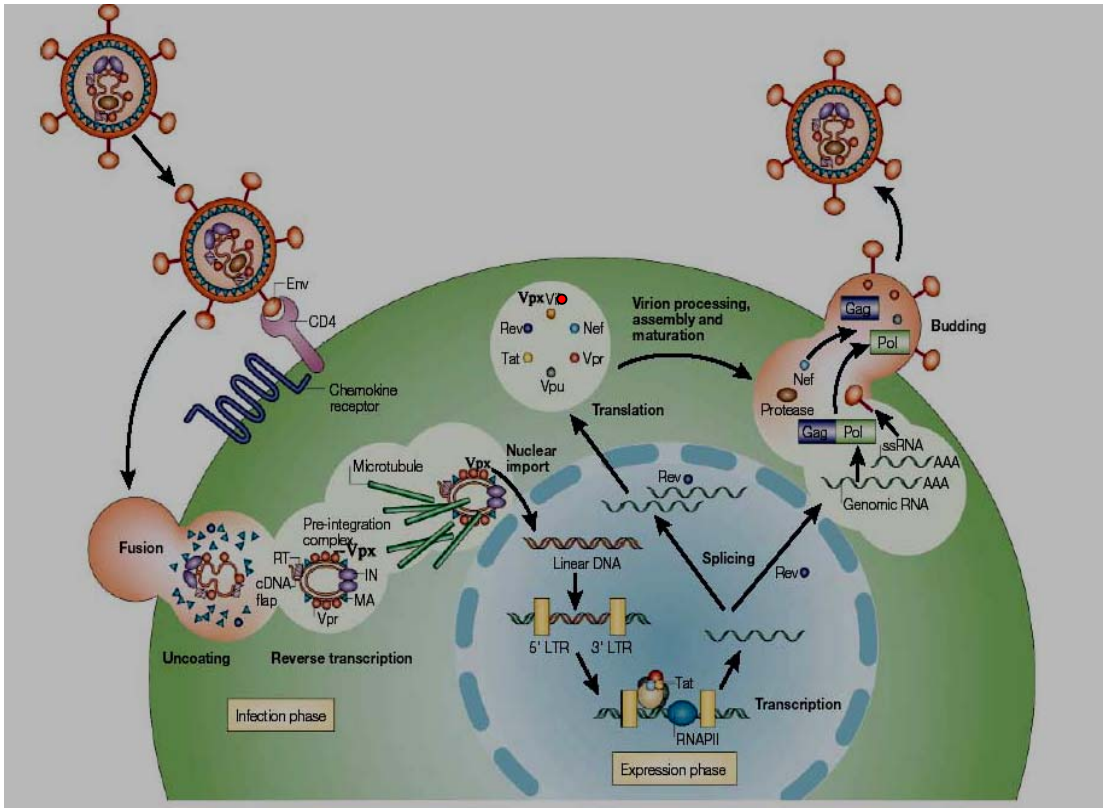


Figure 6: The replicative cycle of HIV.

DNA flap to the nuclear uptake of the PIC (Dvorin *et al*., 2002). Incase of HIV-2/SIVsm viruses SIVsm *vpx* mutant virus has shown severe reduction in

its ability to infect non-dividing cells even in the prescence of MA and Vpr, indicating that Vpx is the most critical determinant for HIV-2/SIVsm infection in non-dividing cells.

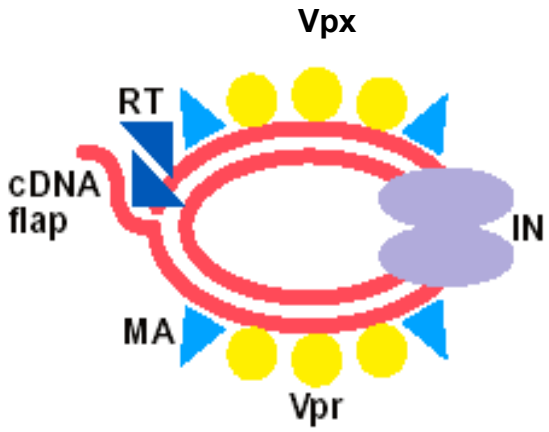


Figure 7: Schematic representation of HIV/SIV preintegration complex. MA (Gag matrix, (Vpr) viral protein R, (RT) reverse transcriptase, (IN) integrase, (Vpx) viral protein X.

2.4.2 Integration.

Integration of double-stranded viral DNA into the host chromosome is mediated by integrase, which binds to the ends of the viral DNA (Miller *et al*., 1997). The host proteins HMGI(Y) and barrier to auto integration factor (BAF) are required for efficient integration (Chen and Engelman, 1998), although their precise functions remain unknown. Integrase removes terminal nucleotides from the viral DNA, producing a two-base recess and thereby correcting the ragged ends generated by the terminal transferase activity of reverse transcriptase (Miller *et al*., 1997). Integrase also catalyzes the subsequent joining reaction that establishes the HIV provirus within the chromosome.

2.4.3 Transcriptional controls.

Integration can lead to latent or transcriptionally active forms of infection. The transcriptional activity of the provirus depends on the chromosomal environment where it is integrated (Jordan *et al*., 2001). In the host genome, the 5´ LTR functions like other eukaryotic transcriptional units. It contains downstream and upstream promoter elements, which include the initiator (Inr), TATA-box (T), and three Sp1 sites. These regions help to position the RNA polymerase II (RNAPII) at the site of initiation of transcription and to assemble the preinitiation complex (Fig. 8). Slightly upstream of the promoter is the transcriptional enhancer, where nuclear factor [kappa] B (NF-κB), nuclear

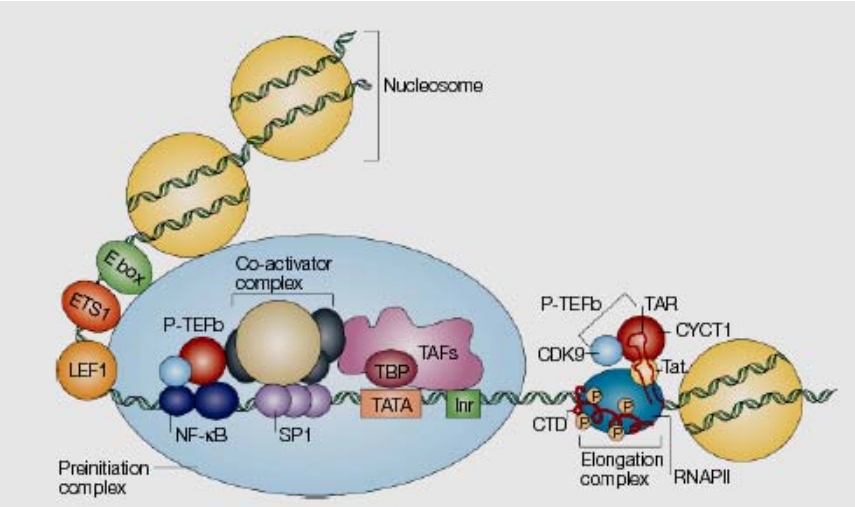


Figure 8: **The HIV long terminal repeat**. The viral promoter contains proximal core and distal upstream promoter elements, as well as enhancer sequences and the transactivation element (TAR) element. The core promoter consists of the initiator (inr) and TATA box, TATA binding protein and (TBP) and TBP-associated factors (TAFs) bind the core promoter. They are flanked upstream by three SP1-binding sites and downstream by TAR RNA structure. The co-activator complex binds SP1, and together, they recruit and position RNA polymerase (RNAPII) in the preintegration complex on the HIV long terminal repeat (LTR).

factor of activated T cells (NFAT), and Ets family members (Jones and Peterlin, 1994) bind. When these factors engage the LTR, transcription begins

but in the absence of Tat, RNA polymerase II fails to elongate efficiently along the viral genome. In the process, short non polyadenylated transcripts are synthesized which are stable and persist in cells due to the formation of an RNA stem loop called the transactivation response (TAR) element (Kao *et al*., 1987). Tat binds to the TAR RNA stem-loop structure and recruits the cellular cyclin-dependent kinase 9 (Cdk9) to the HIV LTR (Wei *et al*., 1998). Within the positive transcription elongation factor b (P-TEFb) complex, Cdk9 phosphorylates the C-terminal domain of RNAPII, marking the transition from initiation to elongation of eukaryotic transcription (Zhou *et al*, 2000).

2.4.4 Viral transcripts.

Transcription of the viral genome results in more than a dozen different HIV-specific transcripts (Saltarelli *et al*., 1996). Some are processed cotranscriptionally and in the absence of inhibitory RNA sequences (IRS), transported rapidly into the cytoplasm (Cullen, 1998). These multiply spliced transcripts encode Nef, Tat, and Rev, other singly spliced or unspliced viral transcripts remain in the nucleus and are relatively stable. The unspliced viral genomic RNAs are needed for the assembly of fully infectious virions. Incomplete splicing results from sub optimal splice donor and acceptor sites in viral transcripts. In addition, the regulator of virion gene expression, Rev, may inhibit splicing by its interaction with alternate splicing factor/splicing factor 2 (ASF/SF2) (Powell *et al*., 1997) and its associated p32 protein (Luo and Peterlin, 1994).

2.4.5 HIV replication.

In contrast to Tat and Rev, which act directly on viral RNA structures, Nef modifies the environment of the infected cell to optimize viral replication. The absence of Nef in infected monkeys and humans is associated with much slower clinical progression to AIDS (Kestler *et al*., 1991; Deacon *et al*., 1995). This virulence caused by Nef appears to be associated by its ability to affect

signaling cascades, including the activation of T-cell antigen receptor, (Simmons *et al*., 2001) and to decrease the expression of CD4 on the cell surface (Khan *et al*., 1998; Glushakova *et al*., 2001). Nef also promotes the production and release of virions that are more infectious (Lama *et al*., 1999; Zheng *et al*., 2001). Other viral proteins also participate in the modification of the environment in infected cells. Rev-dependent expression of Vpr induces the arrest of proliferating infected cells at the G2/M phase of the cell cycle (Jowett *et al*., 1995). Since the viral LTR is more active during G2, this arrest likely enhances viral gene expression (Goh *et al*., 1998).

2.4.6 Viral assembly and budding.

New viral particles are assembled at the plasma membrane. Each virion consists of roughly 1500 molecules of Gag and 100 Gag-Pol polyproteins, (Wilk *et al*., 2001) two copies of the viral RNA genome, Vpx (in HIV-2 and SIVsm) (Henderson *et al*., 1993; Kappes *et al*., 1993) and Vpr (Freed, 1998) Several proteins participate in the assembly process, including Gag polyprotein and Gag-Pol, as well as Nef and Env. A human ATP-binding protein, HP68 likely acts as a molecular chaperone, facilitating conformational changes in Gag needed for the assembly of viral capsids (Zimmerman *et al*., 2002). The Gag polyproteins are subjected to myristylation, (Gottlinger *et al*., 1989) and thus associate preferentially with cholesterol and glycolipid-enriched membrane micro domains (Ono and Freed, 2001). Virion budding occurs through these specialized regions in the lipid bilayer, yielding virions with cholesterol-rich membranes (Fig. 9). This lipid composition likely favors release, stability, and fusion of virions with the subsequent target cells (Campbell *et al*., 2001).

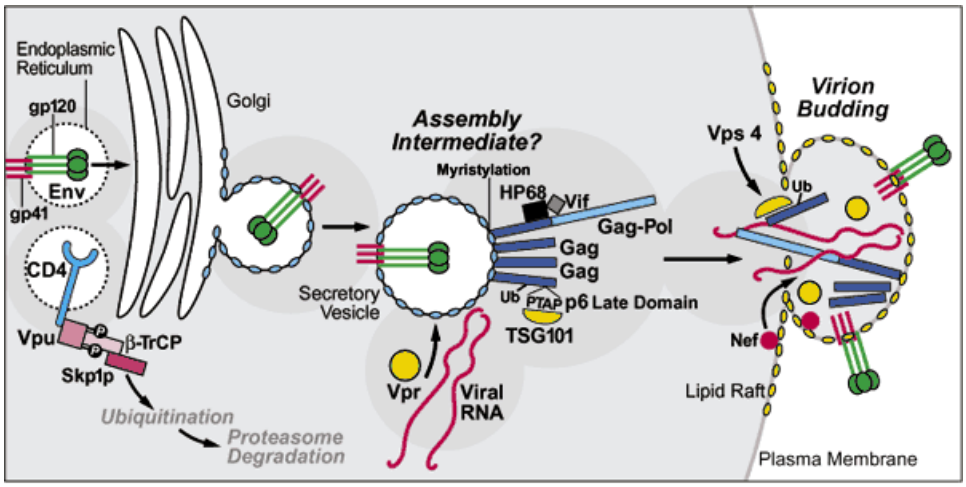


Figure 9: **Late steps in the assembly of new virions**: Components of protein sorting machinery, TSG 101 and Vps4 have critical roles in the terminal phases of virion budding. Although virion assembly occurs principally at the plasma membrane, the involvement of the vesicular sorting proteins indicates that virion assembly may be initiated on secretory vesicles that are destined for the plasma membrane.The final phase of virion release likely involves retargeting of budding away from cytoplasm that normally occurs in the multivesicular body.

The budding process involves the action of several proteins, including the "late domain" (Garnier *et al*., 1999) sequence (PTAP) present in the p6 portion of Gag (Strack *et al*., 2000). The p6 protein also appears to be modified by ubiquitination. The product of the tumor suppressor gene 101 (TSG101) binds the PTAP motif of p6 Gag and also recognizes ubiquitin through its ubiquitin enzyme 2 (UEV) domain (Garrus *et al*., 2001; VerPlank *et al*., 2001).

2.5 HIV pathogenesis.

As intracellular parasites, all viruses must be intimately familiar with host cellular machinery and capable of suborning it to support their replication cycle. For HIV, this relationship is particularly complex and intimate because HIV targets, infects, and incapacitates cells central to antimicrobial defenses. Thus, host immune defenses and HIV pathogenesis are inextricably linked. Whereas this parasitic relationship may contribute to the persistence and progression of HIV infection, careful study of the relationship between HIV and the immune system has also yielded important insights into mechanisms of

immune homeostasis and host. AIDS is essentially an infection of the immune system and the principal impact of HIV infection on the immune system is destruction of CD4 T lymphocytes and other cells of the immune system. The loss of CD4 population ultimately leads to the inability of the infected person to deal with opportunistic infections. In the 2-3 week incubation period that follows sexual mucosal or by parenteral routes, the virus becomes well established in the lymphatic tissue reservoirs (Haase, 1999). This reservoir is the principal site of virus production, storage and persistence (Tenner-Racz *et al*., 1986; Biberfield *et al*., 1986; Pantaleo *et al*., 1991; Fox *et al*., 1993; Pantaleo *et al*., 1993). Progression of disease depends on host genetic factors like HLA polymorphism and HIV co-receptor polymorphism. HLA alleles like B57, B14, C8 and B27 have been shown to be associated with long term non-progressors (Klein *et al*., 1998; McNeil *et al*., 1996; Hendel *et al*., 1999), Whereas HLA B3501 and Cw4 have been consistently associated with accelerated disease progression (Tomiyama *et al*., 1997). Innate immune responses play a role in the progression of the disease but adaptive immune response is the most critical component of immune system for the control of HIV infection.

Two cell populations, plasmacytoid dendritic cells (PDCs) and natural killer cells play predominant roles in innate immune response. Loss of plasmacytoid dendritic cells is reflected by decreased interferon-α production and is associated with higher HIV RNA levels and quicker progression to AIDS (Soumelis *et al*., 2001). PDCs not only participate in the innate immune response through interferon production, they may also become mature dendritic cells and participate in adaptive immune response. Natural killer cells have the natural capacity to kill infected cells in the absence of prior sensitization and without MHC restriction. NK cells display a decreased lytic activity in HIV infected patients, this effect appears to be associated with low

expression of natural cytotoxicity receptors (NCR) on HLA-DR+ NK cells and reduction in expression of perforin molecules (Fogli *et al*., 2004).

HIV affects cells of adaptive immune response like CD4 T helper cells, CD8 cytotoxic T lymphocytes (CTLs) and production of neutralizing antibodies. Antibodies in the sera of HIV infected patients have only weak neutralizing activity for primary HIV isolates (Montefiori *et al*., 1996; Moog *et al*., 1997; Moore *et al*., 1995), with most of the antibodies being non-neutralizing and directed against virion debris (Poignard *et al*., 1999). In addition, the burst of HIV replication that occur in the early days after initial infection is contained in the infected individuals well before the development of an antibody that can neutralize the virus (Pilgrim *et al*., 1997).

Apoptosis is one of the mechanisms that contribute to T cell destruction. CD4 and CD8 T lymphocytes from infected individuals have been found to undergo spontaneous apoptosis *in vitro* (Meyaard *et al*., 1992). HIV proteins Nef, Tat and Env up regulate CD95 and FasL, triggering apoptosis. Vpr also contribute to the direct killing of infected cells by promoting apoptosis (Stewart *et al*., 1997; Somasundaram *et al*., 2002). CD4 T cells produce an array of cytokines that are very important in activating and maintaining effector functions like IL2, IFNγ, IL12 (pro-regulatory cytokines) and IL4, IL10, IL5, IL13 are pro-inflammatory cytokines. A strong CD4 T lymphocyte response in primary HIV infection was found to correlate with slow disease progression and better control over viremia (Gloster *et al*., 2004).

HIV also stimulates strong immune responses by CTLs in infected people. The central role of CTLs in controlling the virus is also emphasized by the influence of HLA type. The loss of CTL function is due to reduced number of CD4 T helper cells, as it is known that CD4 T helper cells are important for priming CD8 T cell responses (Ridge *et al*., 1998), for maintaining CD8 T cell

memory (Walter *et al*., 1995) and for maturing CD8 T cell function (Zajac *et al*., 1998). Cultured HIV-1 specific CTLs have been shown to lyse infected CD4 T cells *in vitro* (Yang *et al*., 1996), whereas virus specific CTLs taken ex vivo have been shown to have functional defects like low levels of perforin that undermine the control of the virus (Zajac *et al*., 1998; Kalams and Walker, 1998; Appay *et al*., 2000).

Once the CD4 count drops less than 200 cells/µl the patients are at high risk for developing opportunistic infections. Different malignancies have been reported, the most common are Pneumocystis carinii pneumonia, tuberculosis, oral hairy leukoplakia and oral candidiasis and prolonged diarrhea. Oral candidiasis has been reported as the most common HIV associated condition occuring up to 70 percent cases. HIV also affects the central nervous system causing opportunistic infections like toxoplasmosis and non-hodgkins lymphoma. Before the availability of anti-retroviral therapy the median survival period after diagnosis of AIDS was 12 to 18 months, but after the advent of highly activated antiretroviral therapy (HAART) the life span of the AIDS patients has increased considerably.

2.6 Nucleo-cytoplasmic transport.

In Eukaryotic cells, the nucleus is separated from the cytoplasm by a double layered membrane known as nuclear envelope (NE). The DNA replication and RNA biogenesis occurs in the nucleus, whereas protein synthesis occurs in the cytoplasm. Eukaryotic cells have evolved mechanisms for integration of these activities by selective transport of proteins and ribonucleoprotein particles between the two compartments. Transport across the NE occurs through the large multiprotein structures, termed nuclear pore complexes. The transport is signal mediated and requires both energy and soluble factors including shuttling carriers (Gerace, 1995; Goldberg and Allen, 1995; Gorlich and Mattaj, 1996; Izzauralde and Mattaj, 1995; Pante and Aebi,

1995). Biochemical and genetic analysis has shown that the basic mechanisms of nucleocytoplasmic transport have been highly conserved during evolution (Powers and Forbes, 1994; Koepp and Silver, 1996).

2.6.1 The nuclear pore complex and nucleoporins.

Nuclear pore complexes (NPCs) mediate bidirectional transport between the cytoplasm and nucleus (Feldherr and Akin, 1990). They provide aqueous channels of about 9nm in diameter, which allows diffusion of ions, metabolites and small proteins of relative molecular mass less than 40K-50 kDa and mediate the selective transport of particles up to 26-28nm in diameter by energy dependent mechanisms (Pante and Aebi, 1995; Davis, 1995). By electron microscopy, NPCs appear as roughly cylindrical structures, with eight fold rotational symmetry in the plane of nuclear envelope (Goldberg and Allen, 1995; Pante and Aebi, 1995; Hinshaw *et al*., 1992; Akey and Radermacher, 1993). Each NPC consists of a basic framework (a spoke complex embracing a central channel) positioned between a cytoplasmic ring and a nuclear ring. The cytoplasmic ring is decorated by eight fibrils, and a basket-like assembly is attached to the nuclear ring. The NPCs may contain approximately 1000 proteins, with multiple copies of some 50-100 different proteins (Bastos *et al*., 1995), and these proteins are collectively called nucleoporins. Many nucleoporins display highly repetitive motifs conforming to either the consensus FXFG and/or GLFG. These FG repeats interact with transport factors (Radu *et al*., 1995; Rexach and Blobel, 1995; Stutz, 1996). These nucleoporins also contain heptad repeats favouring α-helical coiled coil formation and these may promote the assembly of nucleoporin complexes (Buss and Stewart, 1995; Grandi *et al*., 1995; Grandi *et al*., 1995; Hu *et al*., 1996).

2.6.2 Signals for import and export.

An essential demand on the nuclear transport system is the selectivity and specificity, the system has to ensure that only the 'correct' cargoes are imported and exported at the proper time. Specific signals contribute crucially to the fidelity of the nuclear transport. These are in general characterized by the presence of basic residues in one or two clusters, known as monopartite or bipartite (Dingwall and Laskey, 1991). The first NLS was identified in SV40 large T antigen known as classical NLS consisting of a single stretch of basic amino acid residues. Other classes of NLS containing non-basic amino acid residues like the glycine-rich M9 NLS found in hnRNPA1 (heterogenous nuclear ribonucleoprotein A1) (Siomi and Dreyfuss, 1995).

The first nuclear export signal (NES) was identified in HIV-1 Rev protein (Fischer *et al*., 1995) and in a polypeptide inhibitor PKI of the cAMP dependent protein kinase (PKA) (Wen *et al*., 1995). Rev was implicated in the export of viral RNA, whereas PKI functions in the termination of signaling. The prototypic NESs of Rev and PKI are short and hydrophobic with high leucine content. Leucine-rich NES were also found in several other cellular proteins like MEK (Fukuda *et al*., 1996), FxMR1 (Fridell *et al*., 1996), Zxyin (Nix and Beckerle, 1997) and in tumor suppressor BRCA1 (Rodriguez and Henderson, 2000).

The signals specifying transport of RNA's are not well defined but generally believed to reside with RNA associated proteins. In the case of mRNA, export signals appear to be associated with heterogeneous nuclear ribonucleoprotein complexes (hnRNP) which shuttles rapidly between nucleus and cytoplasm and its transport in both directions depends on a 38 amino acid motif which is glycine-rich, indicating that both import and export signals are interdigitated (Siomi and Dreyfuss, 1995; Michael *et al*., 1995; Weighardt *et al*., 1995).

2.6.3 Mechanism of nuclear import.

A major break through for the study of nuclear import was the development of efficient *in vitro* systems (New Meyer *et al*., 1986; Adam *et al*., 1990), which allow the characterization of several transport factors. The initial cytoplasmic event in the NLS mediated nuclear import is the binding of the import substrate to importin-α at the NLS binding site (Adam and Adam, 1994). Importin-α then interacts with importin-β with its importin-β binding domain (IBB) (Gorlich *et al*., 1996; Weiss *et al*., 1996). Importin-β strengthens the affinity of the complex and mediates the docking of the cargo-carrier complex to the NPC (Doye and Hurt, 1995; Gorlich *et al*., 1995a; Imamoto *et al*., 1995). The cargo carrier complex initially contacts the NPC at the distal end of cytoplasmic fibrils, from where it is transferred to the cytoplasmic entry of the central channel (Pante and Aebi, 1996). The transfer of the trimeric NLS/importin α/β complex through the NPC is energy dependent and appears to require GTP hydrolysis by Ran (Melchior *et al*., 1993; Moore and Blobel, 1993). Translocation into the nucleus is terminated at the nuclear side of the NPC by disassembly of the trimeric NLS/importin α/β complex. The NLS/ importin α behaves like an inert cargo during the import and the actual movement through the NPC is generated by importin-β. Once inside the nucleus the importin-α/β heterodimer is dissociated by direct binding of RanGTP to importin-β (Fig. 10) (Rexach and Blobel, 1995; Chi *et al*., 1996; Gorlich *et al*., 1996c). Ran itself is predominantly, though not exclusively nuclear (Bischoff and Ponstingl, 1991b). Ran's major nucleotide exchange factor is the chromatin bound RCC1, which generates RanGTP inside the nucleus (Oshtubo *et al*., 1989; Bischoff and Ponstingl, 1991a). In contrast, Ran's principal GTPase activating protein, RanGAP1, is excluded from the nucleoplasm (Hopper *et al*., 1990; Matunis *et al*., 1996; Mahajan *et al*., 1997). RanGAP1 stimulates conversion of RanGTP to the GDP bound form (Bischoff *et al*., 1994) and thereby depletes RanGTP from cytoplasm. This reaction is

further facilitated by another cytoplamic factor, the Ran-binding protein, RanBP1 (Coutavas *et al*., 1993; Bischoff *et al*., 1995; Richards *et al*., 1996). This asymmetric distribution of Ran, RCC1, RanGAP1 and RanBP1 results in a steep GTP gradient across the nuclear envelope with high nuclear concentration and very low level in the cytoplasm, which allows the importin heterodimer formation in the cytoplasm and forces its dissociation in the nucleus.

2.6.4 Mechanism of nuclear export.

The export of importin-α to the cytoplasm requires a specific nuclear export factor called CAS, this receptor interacts with RanGTP in order to bind to importin-α (Gorlich *et al*., 1997; Izaurralde *et al*., 1997). Both CAS and importin-β have separate binding sites for importin-α and RanGTP. However RanGTP regulates importin-α binding to CAS and importin-β in exactly opposite manner, RanGTP dissociates importin-α from importin-β and it increases CAS' affinity for importin-α by 300 fold (Kutay *et al*., 1997b). The RanGTP gradient across the NE with a high nuclear concentration would thus ensure that importin-α binds tightly to importin-β in the cytoplasm, but preferentially to CAS inside the nucleus. Once the trimeric RanGTP /CAS/importin-α complex has formed it is transferred to the cytoplasm. The cytoplasmic release is accomplished by RanBP1 which initially binds to Ran in the trimeric RanGTP/CAS/importin-α complex (Bischoff and Gorlich, 1997). A RanBP1/RanGTP complex is transiently released and attacked by RanGAP1, which triggers GTP hydrolysis and makes its dissociation irreversible. The Ran free CAS now in the low-affinity form for importin-α binding, releases the cargo re-enters the nucleus to mediate another round of export. Other major export factors identified till to date are an importin-β- like protein, the exportin-t which specifically exports only the mature t-RNA (Arts *et al*., 1998; kutay *et al*., 1998).

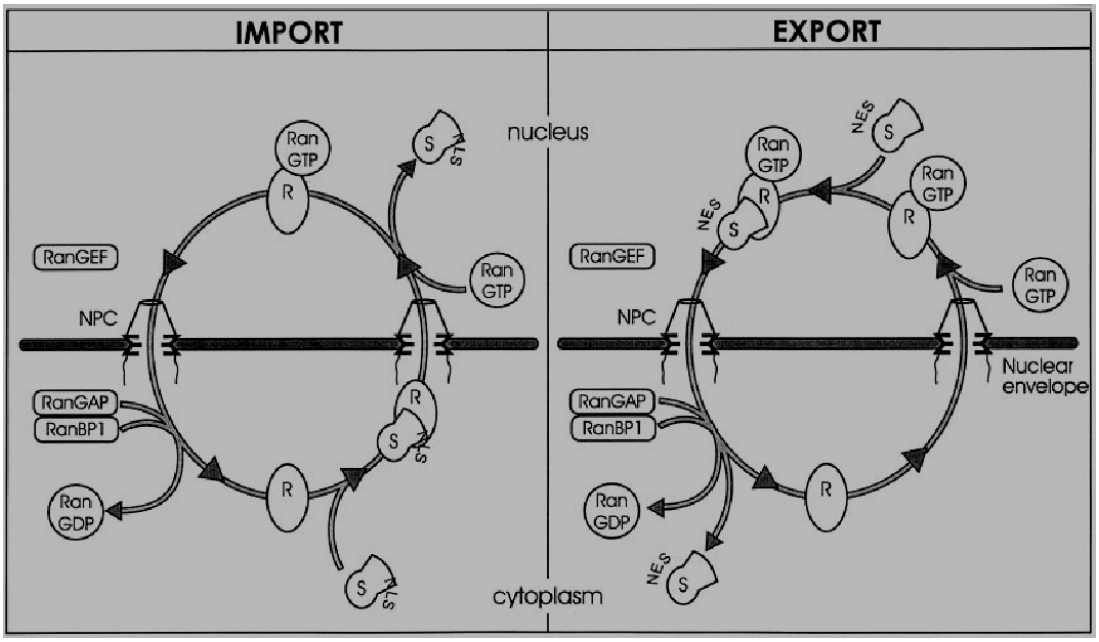


Figure 10: Mechanism of nuclear import and export. Nuclear import and export are mediated by shuttling receptors (R) which recognize nuclear localization signals (NLSs) or nuclear export signals (NESs) in cargoes (S). GAP, GTPase-activating protein; RanBP1, Ran-binding protein 1; GEF, guanine nucleotide exchange factor.

Important insights into export mechanism have come from the studies on HIV1-Rev protein (Fischer *et al*., 1994; Fischer *et al*., 1995) and its functional homologue Rex of human T-cell leukemia virus (Bogerd *et al*., 1996). In HIV infected cells Rev is important for cytoplasmic accumulation of incompletely spliced viral pre mRNAs. The first nuclear export pathway to be discovered involved the CRM1 export receptor, exporting proteins containing a hydrophobic nuclear export signal NES (Fornerod *et al*., 1997; Fukuda *et al*., Ossaerh-Nazari *et al*.,1997). Hydrophobic leucine-rich nuclear export signal (NES) present in Rev binds directly to CRM1 and is exported to the cytoplasm. CRM1 mediated export can be blocked by a fungal metabolite leptomycin B (Wolff *et al*., 1997). Shuttling hnRNP proteins, exemplified by a protein A1 of vertebrates (Michael *et al*., 1995, Visa *et al*., 1996) and the structurally related protein, yeast Npl3p (Lee *et al*., 1996; Singleton *et al*., 1995) have been proposed to play a role in mRNA export.

2.6.5 Regulation of nucleo-cytoplasmic transport.

Proper regulation of nucleo-cytoplasmic transport of various cargoes is crucial for maintaining cellular metabolism by distribution of proteins in response to various external stimuli, and spatial distribution of RNA transcripts during early development (Nakielny *et al*., 1997). Nucleo-cytoplasmic distribution of proteins in general is regulated by two mechanisms, one is based on anchoring and release, and the other is masking and unmasking of NLS and NES (Nigg, 1990).

An example of anchoring and release of NLS proteins is sterol regulatory element binding proteins (SREBPs) involved in cholesterol homeostasis. These proteins are inserted into endoplasmic reticulum membrane and their liberation and translocation into the nucleus requires the cleavage of transmembrane domain (Wang *et al*., 1994), and the released SREBP interacts with importin-β before being translocated into the nucleus (Nagoshi *et al*., 1999). Another important example was demonstrated in notch signaling. Notch proteins are crucial transmembrane receptors, involved in development and their activation by ligand binding involves the proteolytic cleavage of the notch intracellular domain (NICD). The released NICD then migrates from plasma membrane into the nucleus to activate downstream target genes (Schroeter *et al*., 1998, Struhl and Adachi, 1998). It has also been demonstrated that hetero- and homo-dimerization of proteins is also involved in the regulation of the nuclear import, like tyrosine phosphorylation of STATs by JAK (Janus kinase) promotes dimerization of STAT in the cytoplasm and translocation into nucleus (Hirano *et al*., 1997). Drosophila clock proteins were found to be regulated through mutual interaction of PER (period) and TIM (timeless) which are assembled into heterodimer in the cytoplasm and the resulting dimer then migrates to the nucleus, whereas TIM and PER accumulate in the cytoplasm when expressed separately (Saez and young, 1996).

The most common mechanism involved in the regulation of the nucleo-cytoplasmic transport of proteins is phosphorylation/dephosphorylation. Yeast transcription factor SWI5 is located in the cytoplasm when it is phosphorylated near its NLS, but accumulates in the nucleus when dephosphorylated (Moll *et al*., 1991). Alternatively phosphorylation/dephosphorylation also causes conformational changes exposing an NLS or alters protein-protein interactions as seen in the case of NFκB/Rel transcriptional factors (Baeuerle and Henkel, 1994), where the NLSs are masked by interaction with inhibitors of IκB family and nuclear translocation of NFκB/Rel requires inactivation of IκB, by phosphorylation and proteolysis. Phosphorylation/dephosphorylation plays a crucial role in cell cycle progression. It has been shown that essential cell cycle regulators such as cyclins and cyclin dependent kinases shuttle continuously between nucleus and cytoplasm and final accumulation determines the cell cycle progression (Hagting *et al*., 1998; yang *et al*., 1998). In G1 phase CDC6 an essential regulator of initiation of DNA replication, is located in the nucleus. CDK2 activation is essential for entry into S phase of the cell cycle, and when cyclin A /CDK2 is activated it phosphorylates CDC6 due to which it re-localizes to cytoplasm in order to prevent re-replication during the S phase (Petersen *et al*., 1999).

2.6.6 Nuclear transport of viruses.

Viruses replicate within living cells and use cellular machinery for the synthesis of their genome and other components. To gain access, they have evolved a variety of elegant mechanisms to deliver their genes into the host cell. Many animal viruses take advantage of endocytic pathways and rely on the cell to guide them through a complex entry and uncoating program. To infect a target cell, a virus particle proceeds through multistep entry process, during which each step is preprogrammed and tightly regulated in time and space.

Many viruses depend on nuclear host factors for genome replication and, thus at some stage, the viral genome must enter the nucleus (Whittaker and Helenius, 1998). In dividing cells, the genome may become trapped during reconstitution of the nucleus in late telophase of mitosis (Roe *et al*., 1993). But for successful infection of non-dividing cells, the viruses must transport their genome into the nucleus through nuclear pore complex. To move inside the cell, incoming viruses often exploit the cytoskeleton and cellular motor proteins (Dales and Chardonnet, 1973; Greber *et al*., 1997; Sodelik *et al*., 1997). Transport to the nucleus generally involves the minus-end directed microtubule-dependent motor dynein and its adaptor protein, dynactin. Actin also plays an important role in virus entry. Import of virus and viral capsids occurs through the nuclear pore complexes, for targeting, viruses use nuclear localization signals and cytosolic import receptors. The upper limit in particle diameter for transport through the NPC is 39nm (Pante and Kann, 2002).

The smallest viruses and capsids as well as helical capsids in extended form, can be imported into the nucleus without disassembly or deformation. Among viruses with icosahedral symmetry, parvovirus and the capsids of hepatitis B virus are imported intact (Greber and Fassati, 2003). Larger viruses disassemble to allow the genome to pass through the NPC. Adenovirus binds to Nup214/CAN, a protein located at the base of the filaments extending into the cytosol from the nuclear pore (Greber and Fassati, 2003). Interaction of the bound virus with histone H1 and importins-β and importin-7 induces disassembly of the virus capsid and the viral DNA is imported into the nucleoplasm (Fig.11).

The HSV-1 capsids also bind to the NPCs in an importin-β dependent manner, but the DNA is released through one of the icosahedral vertices of the capsid without further capsid disassembly (Ojala *et al*., 2000; Newcomb *et al*.,

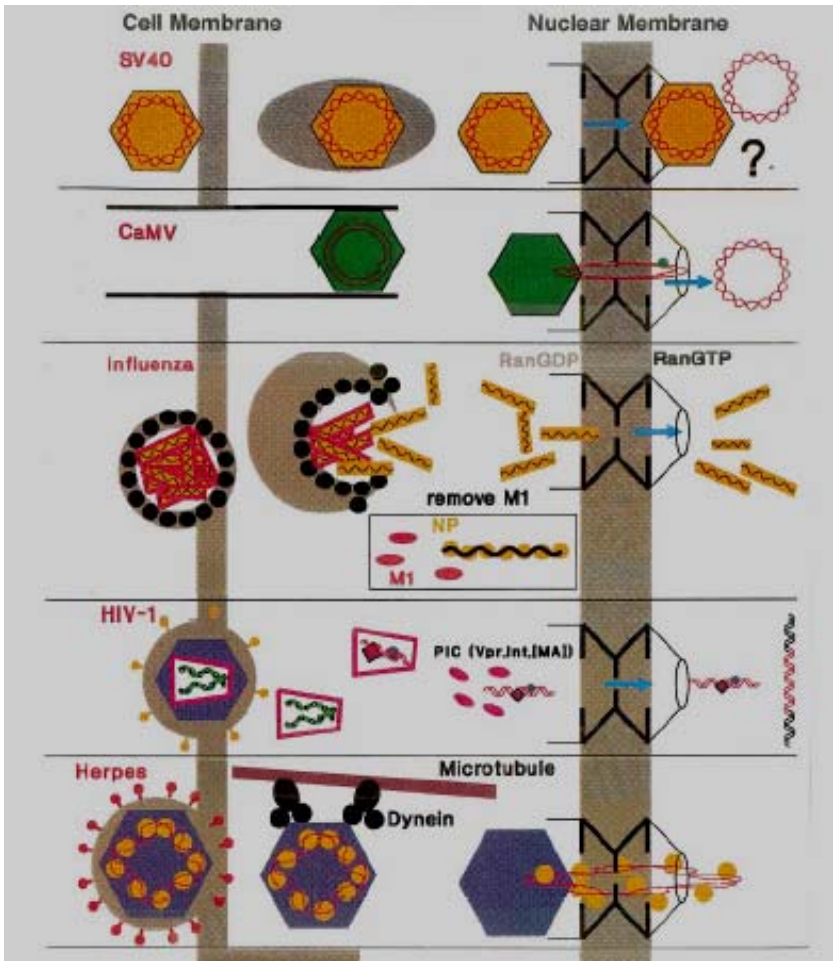


Figure 11: Nuclear import of viral genomes. SV40 virions and influenza virus enter the cell by endocytosis. Cauliflower mosaic virus enters the cell either by insect transmission or from neighboring cells by transport through tubular structures spanning the cell wall. Lentiviruses such as HIV-1 release their nucleocapsids into the cytoplasm where reverse transcription occurs. Herpes virus capsids are transported with the help of dynein along microtubules towards the nucleus, where they release the genome complexed with histone like virus proteins (Izzauralde et al., 1999).

2001). In case of cauliflower mosaic virus CaMV, the NLS which is present adjacent to the N-terminal acidic region is exposed after maturation of the capsid (Leclerc *et al*., 1999). This lysine-rich NLS helps in docking of the capsid to the nuclear pore by utilizing the importin-α and importin-β. In HIV three different proteins namely Matrix, Integrase, Vpr and a cis-acting element,

the central triple helical DNA domain function in a redundant manner to transport the viral genome into the nucleus. Whereas in case of HIV-2/SIVsm, Vpx has been shown to be the major determinant in the nuclear transport of viral DNA. But the exact mechanism(s) involved in the transport of HIV-2/SIV genome into the nucleus in non-dividing target cells is not known.

Chapter Three

Materials and Methods

3.1 Reagents and chemicals:

3.1.1: Table1: Reagents and sources.

Material	Source
Molecular Biology reagents	Sigma, St louis, USA; Merck, Germany; Amersham Biosciences, UK; Boehringer Manheim, Germany
Cloning Vectors	
pCDNA3.0, pCDNA 4.0 His MAX	Invitrogen, Carlsbad, CA, USA
pET 16b, pET 41b	Novagen, Germany
pSK+	Stratagene, USA
Tissue culture reagents	
DMEM; DMEM phosphate free, DMEM –met –cys; Trypsin-EDTA; Antibiotic and Antimycotic; FBS lipofectin; OPTI-MEM	Invitrogen, Carlsbad, CA, USA
Protein Kinase Inhibitors	Calbiochem, USA; Sigma, St. Louis, USA
Cells and Cell Lines	
CEMx174, 293-T HeLa, Cos-7,Vero,Jurkat, Macaque macrophages	American type culture collection National centre for cell science, Pune, India Prepared from total macaque PBMCs
Restriction endonucleases and DNA modifying enzymes	
Restriction endonucleases, T4 DNA ligase, Dpn1	New England Biolabs Inc, MA, USA
DNA and Protein Standards	MBI Fermentas, Germany; Bio-Rad labs, Hercules, CA, USA

Radio isotopes	
γ-P^{32} ATP, S^{35} Methionine and Cysteine, P^{32} Pi	Board of radiation and Isotope technology, BARC, Mumbai

3.1.2 Table 2: Antibodies.

Vpx, Gag	NIH AIDS research reagent programme
β-Gal	Promega life sciences, Madison, WI, USA
GST	Amersham Biosciences, UK
Flag	Sigma Chemical company, St.Louis, USA
ERK-1/MAPK, ERK-2/MAPK, Lck, NIK, JNK, His	Santa Cruz Biotechnology, Santa Cruz, CA, USA
HA	Clonetech, Palo Alto, CA, USA
mab414	Covance , USA
Goat anti-mouse horse radish peroxidase, Goat anti-rabbit horse radish peroxidase	Southern Biotechnology, USA
Alexa fluor Goat anti-mouse 594 Alexa fluor Goat anti-mouse 488 Alexa fluor Goat anti-rabbit 594 Alexa fluor Goat anti-rabbit 488	Molecular probes Inc., Oregon, USA

3.1.3 Table 3: Composition of common media and solutions.

Luria broth	10g tryptone, 10g NaCl, 5g Yeast extract/ litre
Alkaline lysis Solution I	50 mM glucose, 25 mM Tris-Cl, 10mM EDTA

Alkaline lysis solution II	0.2 N NaOH, 1% SDS
Alkaline lysis solution III	3 M potassium acetate pH 5.5
TB buffer for preparation of competent cells	10 mM PIPES (free acid), 15 mM $CaCl_2.2H_20$, 250 mM KCl, 55 mM $MnCl_2.4H_20$
10X SDS-PAGE running buffer	0.25 M Tris base, 1.92 M glycine, 1% SDS
2XSDS loading dye	100 mM Tris-Cl (pH 6.8), 4% SDS (W/V), 0.2% (W/V) Bromophenol blue, 20%(V/V) glycerol, 200 mM β-mercaptoethanol
1X Western blotting buffer	25mM Tris base, 192 mM glycine, 20% (V/V) methanol (to be added before use)
Phosphate buffered saline (PBS)	137 mM NaCl, 2.7 mM KCl, 10 Mm Na_2HPO_4, 2 Mm KH_2PO_4, pH adjusted to 7.4
PBS-T	PBS + 0.05% Tween 20
Blocking buffer	PBS-T+ 5% fat free milk powder
6X DNA loading dye	0.25% (W/V) bromophenol blue, 0.25% (W/V) xylene cyanol FF, 30% (V/V) glycerol in H2o
50 x TAE (1 litre)	242 g of Tris base, 57.1 ml of glacial acetic acid, 100 ml of 0.5 M EDTA
PEG-$MgCl_2$	40% (W/V) polyethylene glycol (PEG 8000), 30 mM $MgCl_2$
Whole cell lysis buffer	20 mM Tris (pH 7.4), 250 mM NaCl, 2.5 mM Sodium pyro phosphate, 2 mM EDTA, 1mM EGTA, 1 mM NaF, 1 mM β-glycerophosphate, 1% Triton X 100, (1mM PMSF, 2µg/ml Leupeptin, 1µg/ml Aprotinin)

Cytoplasmic extract buffer	10 mM HEPES pH (7.9), 10mM KCl, 1 mM EDTA, 1 mM EGTA and 1.5 mM DTT
Kinase assay wash buffer	20 mM HEPES (pH7.9), 25 mM NaCl, 1 mM DTT
Kinase assay buffer (10X)	200 mM HEPES (pH 7.4), 100 mM $MgCl_2$, 10 mM DTT
Buffers for protein purification	
Lysis buffer (Ni-NTA)	50 mM NaH_2PO_4, 250 mM NaCl, 10 mM imidazole (pH8.0) and 1mg/ml lysozyme
Wash buffer	50 mM NaH_2PO_4, 250 mM NaCl, 30 mM imidazole (pH8.0)
Elution buffer	50 mM NaH_2PO_4, 250 mM NaCl, 250 mM imidazole (pH8.0)

3.2 Competent cells and Transformation.

A single colony of *E.coli* DH5α or BL21 DE3 was inoculated into 5 ml of LB medium and incubated overnight at 37^0C temperature with 200 rpm. Overnight grown culture was reinoculated in 200 ml LB medium and incubated for 24 hrs at 18^0C until the O.D reaches 0.5-0.6. Culture was kept on ice for 10 min and the cells were pelleted at 6000 rpm/4^0C/5 min. The pellet was resuspended in 60 ml of ice cold TB buffer (Table 3). Bacterial suspension was kept on ice for 30 min and was respun at 6000 rpm/4^0C/5 min. Finally the pellet was resuspended in TB buffer containing DMSO (7%). This suspension was further aliquoted into 100 µl volumes and were snap freezed in liquid nitrogen, and stored at -70^0C.

About 10-50ng of DNA or ligation mixture was added into 100 µl of competent cells in 1.5 ml microcentrifuge tubes and incubated on ice for 30 min. The tubes were then transferred to a 42^0C water bath and incubated for

45 sec followed by 2 min on ice. To this 750 µl of LB broth was added and the cells were incubated at 37^0C with shaking for one hour. The cells were then pelleted down, resuspended in 100 µl of fresh LB medium and plated on to LB agar plates containing the appropriate antibiotic.

3.3 DNA Isolation.

Small scale isolation and purification of plasmid DNA was done by alkaline lysis method, as described in Sambrook *et al*., where as for large scale preparation, alkaline lysis followed by PEG purification was used.

3.3.1 Small scale isolation and purification of plasmid DNA (alkaline lysis method).

Bacterial cells containing plasmids were pelleted from overnight grown culture by centrifugation at 6500 rpm/4°C/5 min and the. Pellet was resuspended in 100µl of ice cold alkaline lysis solution 1. Solution 2 (200 µl) was then added and the tube was mixed very gently 3-4 times and incubated at room temperature for 5 min. Solution 3 (150µl) was added and mixed by inverting the tube 3 to 5 times, and was incubated on ice for 10 min. Lysate was spun at 12500 rpm/10min/4°C and the supernatant was transferred to a fresh tube. Equal volume of phenol:chloroform was added to the supernatant and then spun at 12500rpm/12min/4°C. Aqueous phase was taken out in a fresh tube and equal volume chloroform:isoamyalchohol was added, mixed properly and was then spun at 12500rpm/12min/4°C. The upper aqueous phase was collected in a fresh tube and 0.6 volume of isopropanol was added and was incubated at room temperature for 30 min and spun at 12500rpm/RT/20min. The pellet was washed with 70% ethanol and was finally resuspended in 50 µl TE buffer and was stored at -20°C.

3.3.2 Large scale purification of plasmid DNA by PEG method.

Overnight bacterial culture (5 ml) was reinoculated in to 100 ml fresh LB (for high copy number plasmids) or 500 ml LB (for low copy number plasmids) and was incubated at 37°C shaker. After 12-16 hrs the cells were pelleted at 6000 rpm/4°C/5 min. Then standard alkaline lysis method was followed as described above but the volumes of Solution I, II and III were scaled up. The crude plasmid preparation was mixed with equal volume of ice-cold 5 M lithium chloride, mixed well, and the solution was centrifuged at 12000 rpm/ 4°C/30 min. The supernatant was transferred to a fresh tube and equal volume of isopropanol was added and incubated at room temperature for 30 min. The precipitated nucleic acids were recovered by centrifugation at 12000 rpm/RT/20min. Supernatant was discarded and the pellet was washed with 70% ethanol. The Pellet was resuspended in 1 ml TE, mixed with equal volume of phenol:chloroform and was spun at 12500rpm/15min/4°C. Aqueous phase was collected in a fresh tube and equal volume chloroform:isoamyalchohol was added, mixed properly and was spun at 12500rpm/15min/4°C. The upper aqueous phase was collected and 2 volumes of ice-cold ethanol was added and incubated at 4°C for 20 min and spun at 12500rpm/15min/4°C. The pellet was then air dried and redissolved in 0.5 ml of water, to this 0.5 ml of PEG-$MgCl_2$ was added and spun at 12000 rpm/RT/15 min. The pellet was washed with 70% ethanol to remove traces of PEG-$MgCl_2$ and the air dried pellet was finally dissolved in 150 µl of TE.

3.3.3 Spectrophotometric estimation of nucleic acids.

The quantity and purity of nucleic acids was determined by measuring the absorbace at 260 and 280 nm. The concentration of nucleic acids was calculated by taking 1 OD_{260}= 50 µg/ml for DNA, 40 µg/ml for RNA and 33 µg/ml for single stranded oligonucleotides. The purity of nucleic acids was checked by their A_{260}/A_{280} ratio.

3.4 Polymerase chain reaction (PCR).

Amplification of DNA fragments was carried out using Deep vent DNA polymerase. Fifty pico moles of each forward and reverse primers along with 200 µM of each dNTP and 1 U of Deep vent DNA polymerase were used in a 50 µl reaction. The reaction conditions for PCR included denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 1 min for each 1kb of template to be amplified. The amplified product was checked in a agarose gel.

Mutations were introduced into PBj 1.9 *vpx* gene (subcloned as a internal SpeI – Cla I DNA fragment) by quick change directed mutagenesis kit (Stratagene). This reaction was done by taking 50 ng of template, 125 ng of mutant forward and reverse primers and *pfu* DNA polymerase. After the PCR reaction Dpn1 enzyme was added and incubated at 37°C for 1 hr for digesting the methylated parental template. Two µl of this digested product was used for transformation into XL-1 Blue competent cells as described in 3.2. The introduced mutations were confirmed by isolating plasmid by alkaline lysis method followed by sequencing. Mutagenized *vpx* genes were reinserted downstream of cytomegalovirus immediate early (IE)/ T7 promotor in eukaryotic expression vector pCDNA3.0 (Invitrogen). Mutagenized *vpx* genes were reinserted into the PBj1.9 proviral vector using a series of subcloning steps. All introduced mutations were confirmed by DNA sequencing.

3.5 Primers.

3.5.1 Primers for construction of Vpx mutant proviruses.

Primer	Sequence
S2L+ S2L-	'5- GTG AGC CAT GTT AGA TCC CAG GG –3' '5- CCC TGG GAT CTA ACA TGG CTC AC –3'
S13N+ S13N-	'5- CAC CTG GAA ACA ATG GAG AAG AAA-3' '5- GTT TCT TCT CCA TTG TTT CCA GGT G-3'
S52F+ S52F-	'5- GTC TGG CGA AGG TTC TGG GAA TAC TG-3' '5- CAG TAT TCC CAG AAC CTT CGC CAG AC-3'

S63,65A + S63,65A -	‘5-ATG GGG ATG GCA GTC GCA TAC ACT AAA-3’ ‘5- TTT AGT GTA TGC GAC TGC CAT CCC CAT-3’
W24S + W24S -	‘5- GCC TTC GAC TCG CTA GAC AGA-3’ ‘5- TCT GTC TAG CGA GTC GAA GGC-3’
W49S + W49S -	‘5- TTC CAG GTC TCG CGA AGG TCC-3’ ‘5- GGA CCT TCG CGA GAC CTG GAA-3’
W53S + W53S -	‘5- CGA AGG TCC TCG GAA TAC TGG-3’ ;5- CCA GTA TTC CGA GGA CCT TCG-3’
W56S + W56S -	‘5- TGG GAA TAC TCG CAT GAT GAA-3’ ‘5- TTC ATC ATC CGA GTA TTC CCA-3’
W49,53,56S + W49,53,56S -	‘5- CTA ATT TTC CAG GTC TCG CGA AGG TCC TCG GAA TAC TCG CAT GAT GAA ATG GG-3’ ‘5- CCC ATT TCA TCA TGC GAG TAT TCC GAG GAC CTT CGC GAG ACC TGG AAA ATT AG-3’
W99S + W99S -	‘5- GGG GCA GGG GGA AGT AGA CCA GGG CCT-3’ ‘5- GAG GCC CTG GTC TAC TTC CCC CTG CCC C-3’
T17I + T17I -	‘5- GGA GAA GAA ATA ATA GGC GAG G-3’ ‘5- CCT CGC CTA TTA TTT CTT CTC C-3’
T28I + T28I -	‘5- GGC TAG ACA GAA TAG TAG AAG AAA-3’ ‘5- TTT CTT CTA TTC TGT CTA GCC-3’
T67A + T67A -	‘5- GTC AGT CAG CTA CGC CAA ATA CAG ATA-3’ ‘5- GTA TCT GTA TTT GGC GTA GCT GAC TGA C-3’
E30P + E30P -	‘5- AGA ACA GTA CCT GAA ATA AAC-3’ ‘5- GTT TAT TTC AGG TAC TGT TCA-3’
H39L + H39L -	‘5- GCA GTG AAT CTA TTG CCG AGG-3’ ‘5- CCCT CGG CAA TAG ATT CAG TGC-3’
K68A + K 68A -	‘5- AGC TAC ACT AAA TAC AGA TAC-3’ ‘5- GTA TCT GTA TTT AGT GTA GCT-3’
R70A + R70A -	‘5- ACT AAA TAC CAG TAC TTG TGC-3’ ‘5- GCA CAA GTA CTG GTA TTT AGT-3’
L74, I75 S + L74, I75S -	‘5- TAC TTG TGC TCA TCA CAG AAA GCT-3’ ‘5- AGC TTT CTG TGA TGA GCA CAA GTA-3’
H82S + H82S -	‘5- ATG TTT ATG TCG TGC AAG AAA-3’ ‘5- TTT CTT GCA CGA CAT AAA CAT-3’
G86, C87S + G86, C87S -	‘5- TGC AAG AAA TCA TCA AGG TGT TTA-3’ ‘5- TAA ACA CCT TGA TGA TTT CTT GCA-3’
R100A + R100A -	‘5- GGG GGA TGG CAG CCA GGG CCT CCT-3’ ‘5- AGG AGG CCC TGG CTG CCA TCC CCC-3’
P103,106S + P103, 106S -	‘5-CCA GGG TCA CCT CCT TCA CCC CCT CCA-3’ ‘5-TGG AGG GGG AGT AGG AGG AGT CCC TGG-3’
P103, 106Y + P103, 106Y -	‘5-CCA GGG ACA CCT CCT ACA CCC CCT CCA-3’ ‘5- TGG AGG GGG TGT AGG AGG TGT CCC TGG-3’
Y66A +	‘5- TCA GTC AGC GCA ACT AAA TAC AGA-3’

Y66A -	‘5- TCT GTA TTT AGT TGC GCT GAC TGA-3
Y69A + Y69A -	‘5- TAC ACT AAA GCA AGA TAC TTG-3’ ‘5- CAA GTA TCT TGC TTT AGT GTA-3’
Y71A + Y71A -	‘5- AAA TAC AGA GCA TTG TGC TTG-3’ ‘5- CAA GCA CAA TGC TCT GTA TTT-3’
Y66,69A + Y66,69A -	‘5- TCA GTC AGC GCA ACT AAA GCA AGA TAC TTG-3’ ‘5- CAA GTA TCA TGC TTT AGT TGC GCT GAC TGA-3’
Y66,71A + Y66,71A -	‘5- TCA GTC AGC GCA ACT AAA TAC AGA GCA TTG TGC TTG-3 ‘5- CAA GCA CAA TGC TCA GTA TTT AGT TGC GCT GAC TGA-3
Y69,71A + Y69,71A -	‘5- TAC ACT AAA GCA AGA GCA TTG TGC TTG-3’ ‘5- CAA GCA CAA TGC TCT TGC TTT AGT GTA-3’
Y66,69,71A + Y66,69,71A -	‘5- TCA GTC AGC GCA ACT AAA GCA AGA GCA TTG TGC TTG-3’ ‘5- CAA GCA CAA TGC TCA TGC TTT AGT TGC GCT GAC TGA-3
Y66,69,71P + Y66,69,71P –	‘5- TCA GTC AGC CCT ACT AAA CCT AGA CCT TTG TGC TTG-3’ ‘5- CAA GCA CAA AGG TCA AGG TTT AGT AGG GCT GAC TGA-3
Y66,69,71S + Y66,69,71S -	‘5- TCA GTC AGC TCA ACT AAA TCA AGA TCA TTG TGC TTG-3’ ‘5- CAA GCA CAA TGA TCA TGA TTT AGT TGA GCT GAC TGA-3

+, Forward primer; **-**, Reverse primer

3.5.2 Primers for cloning of Vpx deletion constructs.

Primer	Sequence
LacZ-Vpx Nde1/BamH1+	‘5- GGT TTC CAT ATG GGA TCC ATG TCA GAT CCC AGG GAG AGA AT-3’
LacZ/Vpx /BamH1+	‘5- CAA AAA GGA TCC ATG TCA GAT CCC AGG GAG AGA AT-3’
Vpx C20-	‘5- GTC TAG CTC GAG TTA CTC GCC TAT TGT TTC TTC TCC AC-3’
Vpx C40-	‘5- GTC TAG CTC GAG TTA CAA ATG ATT CAC TGC TGC CCT-3’
Vpx C60-	‘5- GTC TAG CTC GAG TTA CAT TTC ATC ATG CCA GTA TTC CC-3’
Vpx C80-	‘5- GTC TAG CTC GAG TTA AAA CAT AGC TTT CTG TAT CAA GC-3’

Vpx C100-	'5- GTC TAG CTC GAG TTA TCT CCA TCC CCC TGC CCC ATG CT-3'
Vpx N 20+	'5- CAA AAA GGA TCC GCC TTC GAC TGG CTA GAC AGA ACA GT -3'
Vpx N40+	'5- CAA AAA GGA TCCC CCG AGG GAA CTA ATT TTC CAG GTC TG-3'
Vpx N60+	'5- CAA AAA GGA TCC GGG ATG TCA GTC AGC TAC ACT AAA TA-3'
Vpx N80+	'5- CAA AAA GGA TCC ATG CAT TGC AAG AAA GGG TGT AGG TG-3'
Vpx N100+	'5- CAA AAA GGA TCC CCA GGG CCT CCT CCT CCT CCC CCT CC-3'

+, Forward primer; **-**, Reverse primer

3.5.3 Primers for cloning Gag deletion constructs.

Primer	Sequence
PBj Gag EcoR1+	'5-GATCCGAATTCTATGGGCGCGAGAAACTCC-3'
PBj Gag Xho1-	'5-GTGGTGCTCGAGTTGGTCTTCTCCAAAGAG-3'
PBj Gag p27 Xho1-	'5- GAT CAG CTC GAG TTA CAT TAG TCT AGC TTT TTG TCC TGG-3'
PBj Gag p17 Xho1-	'5- GAT CAG CTC GAG TTA GTA ATT TCC TCC TTT GCC ACT-3'
PBj Gag p15 BamH1/EcoR1+	'5- ATC GGG GAT CCG GAA TTC ATG GAC GAG GCA TTA AAA GAT-3'
PBj Gag p6 BamH1/EcoR1+	'5- ATC GGG GAT CCG GAA TTC CCC ATG GCC CAG ATG CCT CAG-3'
PBj Gag p15 Xho-	'5- ATG CTA CTC GAG CTA TTG GTC TTC TCC AAA GAG AGA ATT G-3'

+, Forward primer, **-**, Reverse primer

3.6 Cell culture.

293T, Vero, HeLa, Cos-7, and Jurkat cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with pencillin (100 units/ml), streptomycin (100 mg/ml) and 10% fetal bovine serum (FBS). CEMx174 cells were maintained in RPMI 1640 supplemented with 2mM L-glutamine, pencillin (100 units/ml), streptomycin (100 mg/ml) and 10% fetal bovine serum. All the cell lines were maintained in a 37^0C incubator in 5% CO_2.

Macaque peripheral blood mononuclear cells (PBMCs) were obtained from heparin-treated whole blood using lymphocyte separation medium (Oragnon, Teknika). Macrophages were purified from unstimulated macaque PBMCs. Macaque PBMCs ($3x\ 10^{6}$) were placed in 12-well tissue culture plates in macrophage medium containing 10% autologous macaque serum and conditioned medium to supply growth factors. Non-adherent cells were removed after 30-60 min of incubation at 37^0C, followed by extensive washing with phosphate buffered saline (PBS). Cells were allowed to differentiate in macrophage medium for 10-12 days prior to virus infection.

3.7 Transfection.

All the transfections were performed by using a infection-transfection protocol. 293T, Vero, HeLa and Cos-7 cells were grown in DMEM supplemented with 10% FBS. The cells were trypsinized and reseeded in 60 mm petridishes at about 60% confluency 24hr before transfection. Cells were washed twice with PBS and then infected with recombinant vaccinia virus encoding T7 RNA polymerase (vTF7-3), at a multiplicity of infection of 10 for one hour in 5% CO_2 at 37^0C. Mean while DNA: lipofectin mixture was prepared as follows, 10 µl of Lipofectin (Invitrogen, Life technologies) was added to 90µl of opti-MEM and incubated for 45 min at room temperature. Respective plasmid DNA (5-10 µg) was diluted in 100 µl of Opti-MEM. After 45 min the DNA and the Lipofectin were mixed properly and left at room temperature for 15 min for the formation of DNA-Lipofectin complexes. After one hour of infection, the cells were washed twice with sterile PBS. Finally, 200 µl of the DNA-Lipofectin mix was mixed with1.8 ml Opti-MEM, and layered on the top of the cells and further incubated in 5% CO_2 at 37^0C. After 3-4 hrs of incubation equal volume of DMEM with 10% FBS was added and incubated further for 24 hrs. At the end of 24 hrs the cells were harvested in ice-cold PBS in a

microcentrifuge tube by pelleting at 10,000 rpm/ 5min/4°C, the cell pellets were stored at -70^{0}C.

3.8 Generation of virus stocks and infection.

For generation of virus stocks, 293T cells were transfected with various SIVsm PBj1.9 proviral DNAs (10 µg) using effectene transfection kit (Qiagen). Forthy-eight hours after transfection, cell culture supernatants were collected and analyzed for core antigen (p27 gag) content using an SIV core antigen assay (Coulter). These culture supernatants were used in infection experiments.

CEMx174 cells were infected with supernatants containing 10ng of p27gag and incubated overnight at 37^{0}C in 5% CO_2. Infected cells were resuspended in 10 ml of complete T-cell medium and the culture supernatants were collected every three days and were frozen at -70^{0}C for quantification of p27gag at the end of experiments. Terminally differentiated macaque macrophages were infected with virion preparations containing 10ng of p27gag in 12-well plates overnight at 37^{0}C in 5% CO_2 and then washed extensively to remove residual virus. Infected cells were incubated at 37^{0}C. Culture supernatants were collected at three day intervals and frozen at -70^{0}C for p27gag determination.

3.9 Preparation of cytoplasmic and nuclear extracts.

Fresh cell pellet in a micro centrifuge tube was resuspended in 200 µl of ice-cold hypotonic cytoplasmic extraction buffer (Table 3) and incubated on ice for 30 min. To the cell suspension 10µl of 10 % NP40 (freshly prepared) was added and vortexed vigorously for 15 seconds for the lysis of the plasma membrane. The contents were centrifuged for 1 min at 10000 rpm and the supernatant (cytoplasmic extract) and pellet (nuclear fraction) were stored at -70^{0}C.

3.10 Protein estimation by Bradford method.

Protein concentration was estimated by bradford method. The estimation was done at 570nm using BSA (Std 1-50µg/ml; Std 2-100 µg/ml) as standards. The unknown protein concentration was calculated as follows.

$$\frac{\frac{\text{BSA Std 1(Conc)}}{\text{OD}} + \frac{\text{BSA Std 2 (Conc)}}{\text{OD}}}{2} = X$$

X x 50 (dilution factor) / 1000 = Y

Concentration of unknown protein sample (µg/µl) = Y x sample OD.

3.11 Western Blotting.

The proteins were resolved in a denaturing SDS-PAGE gel. Following electrophoresis the proteins were transferred on to Hybond-P PVDF (Amersham biosciences) membrane by electroblotting at 75V for 3 hr. Then the membrane was kept in blocking solution (5% non-fat milk solution in PBS-T) at 4°C overnight. Before probing with primary antibody, the membrane was washed once with PBS-T to remove excess of fat free milk. The membrane was then probed with primary antibody diluted in blocking solution for 3 hours. After incubation the membrane was washed with PBS-T for 30 min (3x 10 min) and further incubated with secondary antibody (conjugated with horse-radish peroxidase) diluted in 5% fat free milk solution (in PBS-T) 45 minutes at room temperature. After incubation the membrane was washed and the protein bands were visualized using enhanced chemiluminescence plus detection system (Amersham Biosciences).

3.12 Immunofluorescence.

Vero and Cos-7 cells in chamber culture slides were infected with vTF7-3 and transfected with various expression plasmids using lipofectin as described section 3.6. Ten to twelve hours after transfection, the cells were fixed with 3% parafomaldehyde and permeabilized with 0.1% Triton X100. Then the cells were probed with the respective primary antibody at 37°C for 90 min. The cells were then washed thrice with PBS and probed with fluorochrome tagged secondary antibody (Fluorescent iso thiocyanate or Alexa fluor 488/594) for 90 min at 37°C. After the incubation the cells were finally washed with PBS and mounted in mounting medium containing 4,6-diamidino-2-phenyl indole (DAPI) to stain the nuclei.

3.13 Metabolic labeling and Immunoprecipitation.

HeLa and Cos-7 cells were transfected with various DNA constructs using infection-transfection protocol mentioned above. Ten to twelve hours after transfection the cells were starved for one hour in serum free medium and then labeled for 6hrs with phosphate free DMEM containing 1 mCi P^{32} orthophosphoric acid, 1% dialyzed FBS or Met/Cys- free DMEM containing 0.3 mCi of S^{35} methionine and cysteine.

After labeling the cells were harvested in cold PBS and pelleted at 10000 rpm/2 min/ 4°C. The cells were then resuspended in 100 µl cell lysis buffer and kept on ice for 30 min with intermittent vortexing. The cell suspension was then spun at 12000 rpm/5 min/ 4°C and the supernatant was collected into fresh microcentrifuge tube. To the cell lysate respective antibodies were added (0.3 µg of antibody for 200µg of cell lysate). Tubes were then kept at 4°C on a rotating platform for 2-3 hrs then 5mg of protein A sepharose (per sample) was added to the antigen-antibody complexes and was incubated further for 90 min. The samples were then run on a SDS 12% PAGE after extensive

washing with lysis buffer. For gels with S^{35} samples, the gels were soaked with 1M sodium salicylate for 15 min, dried and autoradiographed using Kodak X-Omat film.

3.14 *In vitro* kinase assay.

Jurkat cells ($3x10^6$) were treated with 100nm of PMA for 1hr. Cell extracts were prepared by lysing the cells inbuffer containing 20mM HEPES (pH 7.4), 2mM EDTA. 250mM NaCl, 1% NP 40, 2µg/ml leupeptin, 2µg/ml aprotinin, 1mM PMSF. Immunoprecipitation was carried out as mentioned in section 3.13 with specific kinase antibodies. The protein A sepharose bead bound immune complexes were washed extensively with wash buffer (4x400µl) and once with kinase buffer. Kinase assays were performed for 45 min at 30°C with 5 µg of protein in 20mM HEPES pH7.4, 10mM $MgCl_2$, 1mM DTT and 10µCi of γ^{32} ATP. Reactions were stopped by adding 20µl of 2xSDS sample buffer, boiled for 5 min and then resolved on a SDS-15% PAGE.

3.15 Protein purification.

3.15.1 Purification of HIS-tagged proteins by Ni-NTA agarose.

Expression plasmids were transformed into *E.coli* BL21 cells and overnight grown culture (5 ml) of BL21 was inoculated in a 500 ml of LB and was kept in a incubator shaker at 37°C until the O.D reaches 0.5-0.8. The culture was then induced with 1mM IPTG for 4hr. the cells were then pelleted by centrifugation at 6000 rpm/5 min/4°C. The pellet was resuspended in 20 ml lysis buffer (table 3) containing 1 mg/ml lysozyme and protease inhibitors like 1mM PMSF, aprotinin (1µg/ml) and was incubated on ice for 30 min. Sonication was done for 10 min at 20% power output on ice. The lysate was then spun at 12000 rpm/5 min/4°C and the supernatant containing the protein was collected in a fresh tube.

One ml of the 50% Ni-NTA slurry was added to 4 ml of cleared lysate and was mixed at 4°C for 60 min. The lysate-Ni-NTA mixture was loaded into a polypropelene column. The bottom of the column was unplugged and the slurry was washed twice with 3 column volumes of wash buffer (table 3). Finally the proteins were eluted with 0.5 ml of elution buffer. A total of four fractions were collected and the samples were analyzed on a SDS-12% PAGE to check the purity of the protein.

3.15.2 Purification of GST-tagged proteins by GST-sepharose.

Cell lysates were prepared as described in section 3.15 A. One ml of GST-sepahrose beads were taken in a fresh tube and were washed twice with PBS so as to remove ethanol. Then the beads were mixed with cleared lysate and incubated for 1hr on a rocking platform at room temperature. The beads were then washed for 3 times with PBS. Finally the protein was eluted with 3x0.5 ml of reduced glutathione (10mM reduced glutathione in 25 mM Tris buffer pH 8.0. The elution fractions were then resolved on SDS-12 % PAGE and stained with coomasie brilliant blue.

Chapter Four

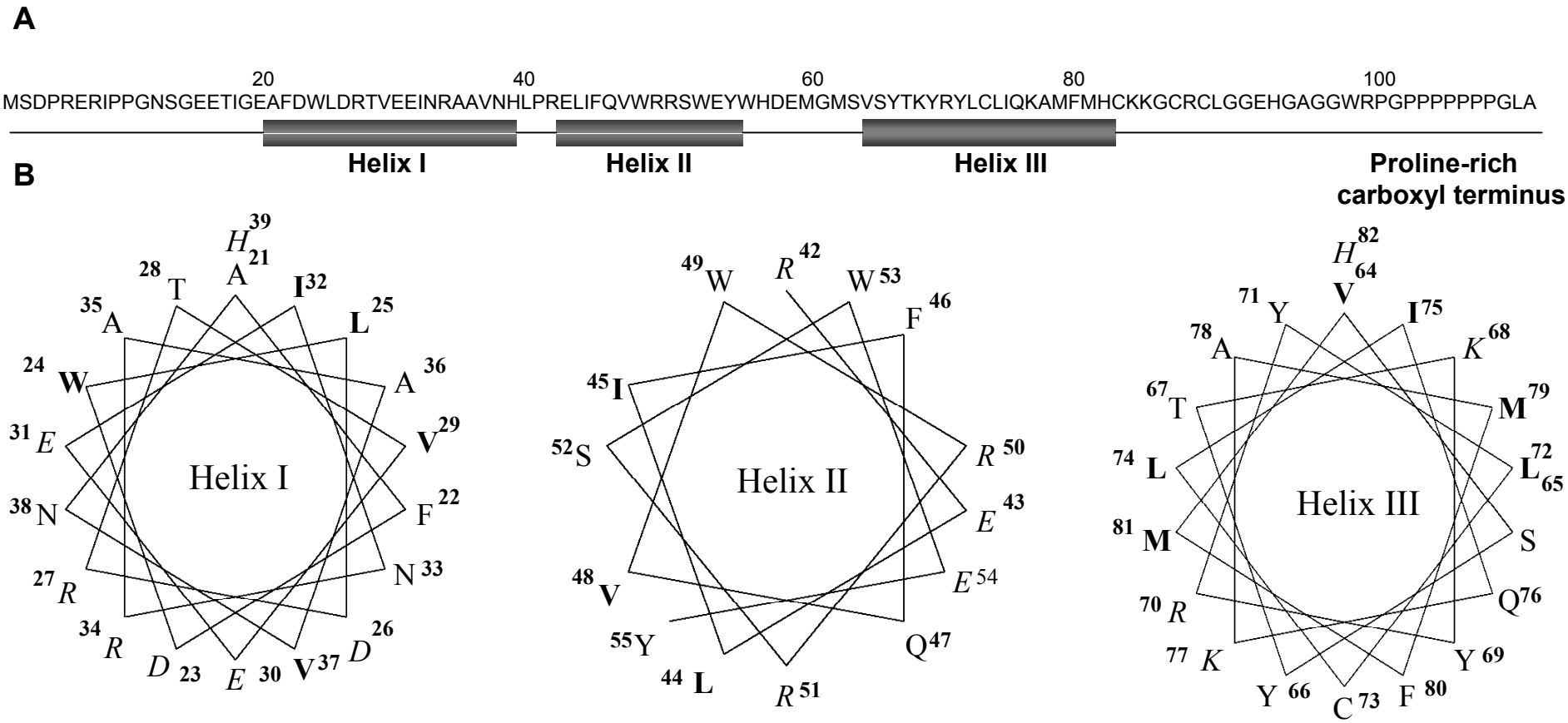
Vpx encodes a novel transferable nuclear localization signal

P.Rajendra kumar, Prabhat K. Singhal, Srinivas S. Vinod and S. Mahalingam. 2003. A Non-canonical transferable signal mediates nuclear import of simian immunodeficiency virus Vpx protein. *J. Mol. Biol*, **331**, 1141-1156.

A critical step in the process of retrovirus infection is the transfer of viral genomic DNA into the nucleus of the infected cells. In HIV-1, the import of viral preintegration complex (PIC) in non-dividing cells is mediated by three different proteins namely Vpr, Gag matrix and integrase in a redundant manner. Whereas in HIV-2/SIVsm, Vpx was found to be the major determinant involved in the nuclear import of viral genome. Vpx is an 18 kDa, 112 amino acid protein, which is highly conserved among all the divergent isolates of HIV-2 and SIVsm. Protein transport is generally considered to involve specific nuclear localization signals (NLS) and proteins that exceed the 35-40 kDa diffusion limit of the nucleopore complex (NPC) must be actively imported via specific pathways following the recognition of NLS. Vpx does not contain sequence elements homologous to any of the previously characterized NLS domains yet it is efficiently transported into nucleus independent of other virus encoded proteins. In this part of the work, we created a number of *vpx* mutants by site-directed mutagenesis to identify the domain(s) involved in Vpx nuclear localization. To distinguish whether Vpx nuclear transport is by passive diffusion or by a signal mediated process and to identify the minimal domain required for Vpx nuclear transport, we constructed chimeric proteins by fusing Vpx with a large cytoplasmic protein, β-Galactosidase (β-Gal).

4.1 Structural features of SIV Vpx.

Vpx is a small, 112 amino acid long protein that is efficiently packaged in the virion in molar amounts equivalent to that of structural protein $pr55^{Gag}$. The predicted secondary structure as indicated by several algorithms showed that Vpx contains three helical domains with a proline-rich C-terminus (Fig. 4.1A). The helical domain I of Vpx comprises amino acid residues 21-39, helical domain II extends from amino acid residues 42 to 55 followed by a helical domain III corresponding to residues 64-82. The detailed predictions and its probability coefficients are indicated in figures 4.1 A, B and C. As can be seen in the scale of 1-10 for helical state, the helix rate of 9 for the vast majority of



C

	Helix I	Helix II	Helix III
AA	AFD**W**LDR**T**V**E**EINRAAVN**H**	RELIFQV**W**RR**SW**EY	V**SYTKYRY**LC**LI**QKAMFM**H**
PHD sec	HHHHHHHHHHHHHHHHHH	HHHHHHHHHHHHHH	HHHHHHHHHHHHHHHHHHH
Rel sec	5899999999999999962	88999977788761	7899999999999999998

Figure 4.1: Secondary structure prediction of SIVsmPBj1.9 Vpx. A. Linear amino acid sequence of Vpx from SIV isolate PBj1.9. **B.** Helical wheel representation to the predicted structure denoting the alpha helical organization of the motifs. Charged residues are shown in italics whereas hydrophobic residues are shown in bold. **C**. Secondary structure prediction. The abbreviations are AA. Amino acid sequence; H-helix; e-extended sheet; Rel, reliability index prediction.

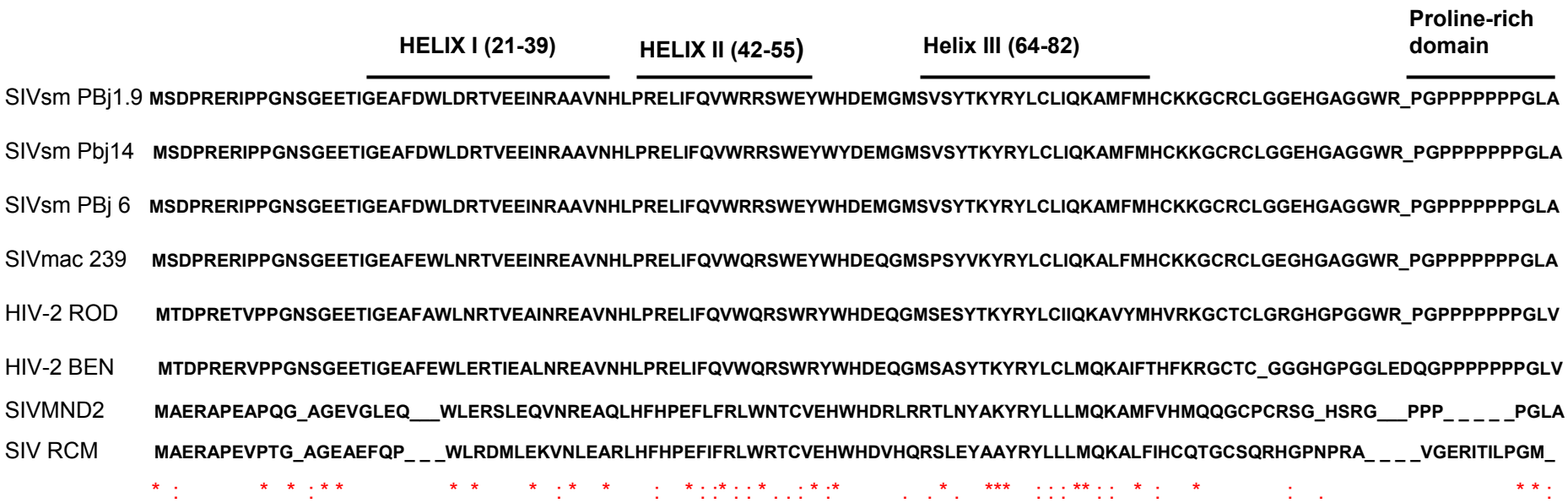


Figure 4.1 D: Alignment of deduced aminoacid sequences from divergent HIV-2 and SIV isolates. Sequences are compared with SIVsmPBj1.9 Vpx sequence. Asterisk denote sequence identity and semicolon indicates similarity. The sequences were obtained from HIV sequence database of Los alamos national laboratory (www.web-lanl.gov) and were aligned with CLUSTALW.

the residues in all three helical domains indicate that these regions have high propensity to form helical structural motifs in Vpx. Multiple sequence analysis of these regions suggests that these structural motifs are conserved across distinct HIV-2 and SIV isolates derived from different consensus groups (Fig. 4.1D). Such conservation of these motifs and its sequences indicate that these domains are important for various functions of Vpx.

4.2 Sub-cellular localization of SIVsm PBj 1.9 Vpx in mammalian cells.

To check the localization of Vpx in mammalian cells, *vpx* gene of infectious molecular clone SIVsmPBj1.9 was taken as a representative of the HIV-2/SIVsm group of viruses. This strain has a complete set of uninterrupted accessory genes, replicates well in macaque macrophages and represents a primary isolate. Using SIVsmPBj1.9 provirus clone as a template, *vpx* gene was PCR amplified as described in materials and methods and cloned into mammalian expression vector pCDNA3.0 (Fig. 4.2A). To check the sub-cellular distribution of Vpx, vTF7-3 infected Vero cells were transfected with Vpx expression plasmid, and the localization was detected by indirect immunofluorescence (Fig. 4.2B) using anti-Vpx monoclonal antibody followed by anti-mouse Alexa fluor-488 conjugated secondary antibody. In order to asses the sub-cellular localization of Vpx in intact cells in the present study, we also used confocal scanning microscopy. Vpx was localized predominantly in the nuclear envelope and inside the nucleus and a certain amount was present in the cytoplasm of the transfected cells (Fig. 4.3). Therefore, it appears that Vpx does not depend on any other viral cofactors for its nuclear localization.

4.3 Construction and expression of mutant *vpx* genes.

In an effort to identify the functional importance of conserved protein structural domains in Vpx, mutations were introduced into the *vpx* gene of infectious molecular clone SIVsmPBj1.9 (Fig. 4.4). Amino acid residues which

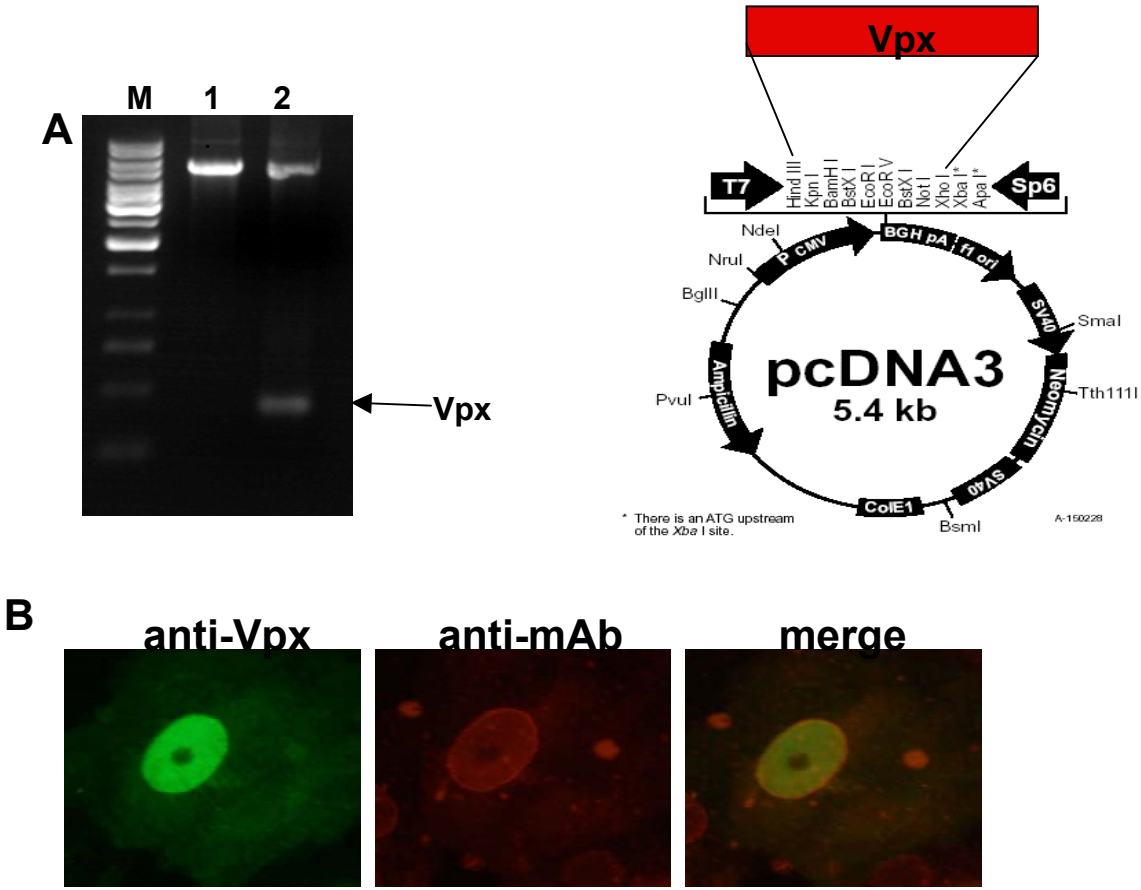


Figure 4.2: Vpx localizes to the nucleus. A. Cloning of *vpx: vpx* was amplified from SIVsmPBj1.9 proviral clone by PCR and cloned into mammalian expression vector pCDNA3.0. (M-1kb DNA ladder; lane 1- linearized pCDNA3.0 vector; lane 2- pCDNA3.0 vector with insert, *vpx*) **B.** vTF7-3 infected Vero cells were transfected with Vpx expression plasmid, and the localization was detected by indirect immunofluorescence using anti-Vpx monoclonal antibody followed by goat anti-mouse Alexa fluor-488 conjugated secondary antibody. Green indicates localization of Vpx and red indicate staining of the nuclear membrane with mAb414 (antibody that recognizes FXFG repeats in nucleoporins in the nuclear membrane).

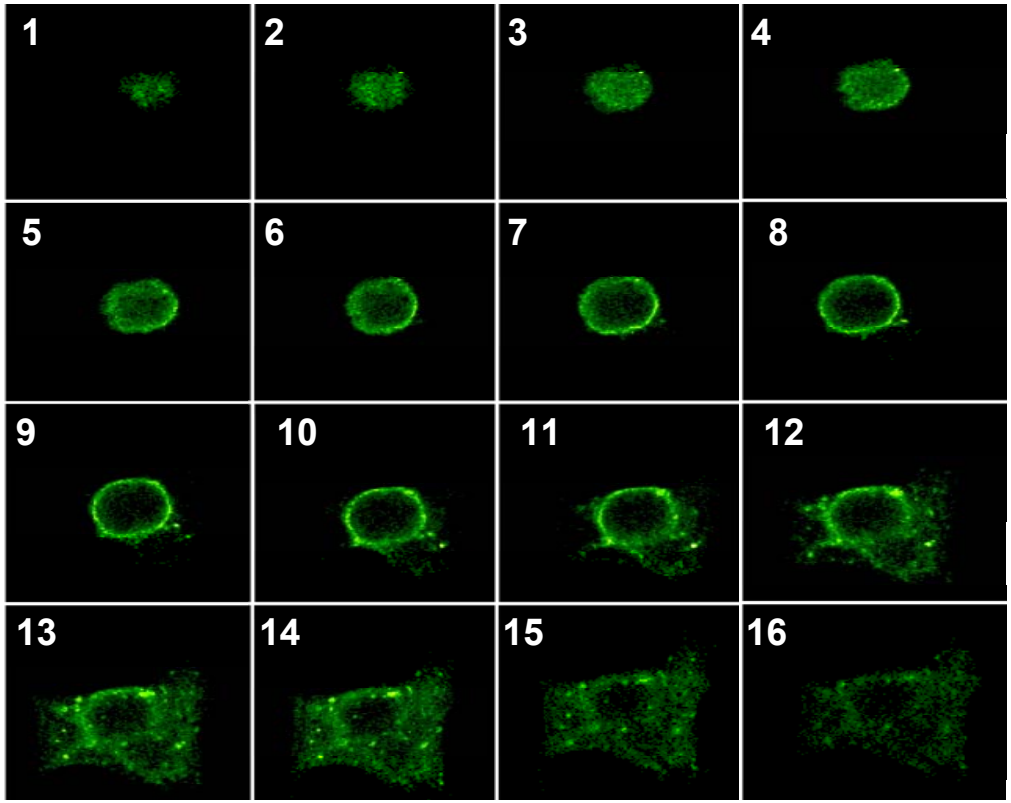


Figure 4.3: Confocal section analysis of wild- type Vpx protein. vTF73 infected Vero cells were transfected with Vpx wild type expression plasmid. Twelve hours after transfection the cells were probed with anti-Vpx monoclonal antibody followed by goat anti-mouse FITC. Photomicrographs were numbered 1, which corresponds to the nuclear periphery through 16 which corresponds to the region through which cell was adsorbed to the slide. The sections were taken at 0.5μm intervals. The sections clearly show that Vpx is localized predominantly on the inner side of nuclear membrane. The staining of cytoplasm becomes more obvious from sections 11-16.

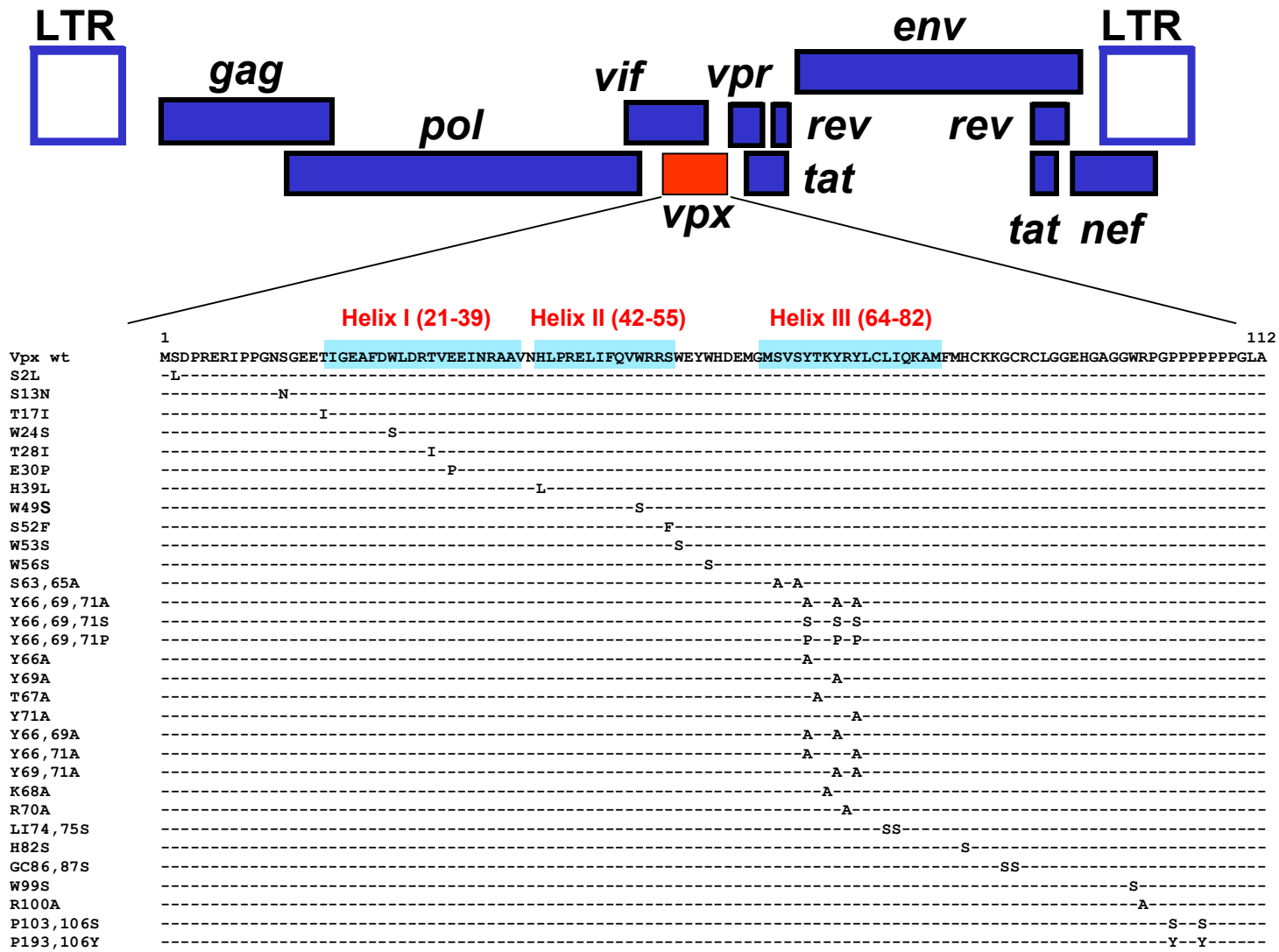


Figure 4.4: Construction of SIVsmPBj1.9 *vpx* mutant proviruses. The genomic organization of SIVsmPBj1.9 is shown at the top, with Vpx substitutions shown at the bottom. Amino acid residues are indicated by standard single letter abbreviations. Amino acid sequences were deduced from the same region after sequencing five different clones of *vpx* and aligned below wild type sequence. Amino acid residues identical to that of wild type protein are indicated by dashes. Names of different modified forms of Vpx are indicated to the left. None of the amino acid substitutions have altered the coding sequences of the overlapping *vif* gene.

are potential sites for phosphorylation like serines (position 2, 13, 52, 63 and 65), threonines (position 17, 28 and 67), and tyrosines (position 66, 69 and 71) were targeted. Phophorylation of such residues in HIV-1 Gag is known to play a critical role in the nuclear import of viral PICs and virus replication in non-dividing macrophages. In addition, other conserved residues such as E30, H39, K68, R70, L74, I75, H82, G86, C87, R100, P103 and P106 of Vpx were also selected for mutagenesis. For most instances, the substitutions were with serine except the substitutions in the N-terminal half of Vpx. Conserved residues at the N terminus of Vpx (aa1-56) where it overlaps with Vif were exchanged with either leucine or phenylalanine in order to maintain the wild-type Vif open reading frame. Proline-rich motifs and tryptophan residues have been shown to mediate protein-protein interactions and implicated in nuclear translocation of viral and cellular proteins (Pancio *et al*., 2000; Garnier *et al*., 1996). In an effort to understand what role, if any, conserved C-terminal proline-rich motif and tryptophan play in Vpx functions, all tryptophans (amino acid positions 24, 49, 53, 56 and 99) were substituted with serines and the prolines at C-terminus were also exchanged with serines and tyrosines separately. As expected, cells transfected with expression plasmids containing wild-type Vpx expressed an 18 kDa protein as seen by Western Blot analysis. This was also the case for cells transfected with all of the mutant expression plasmids (Fig. 4.5 A, 4.6 A, 4.7 A and 4.8 A).

4.4 Mutations in the C-terminal domain affect nuclear import of Vpx.

To identify the functional domains of Vpx that are involved in nuclear localization, a series of plasmids that encode variants of Vpx with substitutions in different regions as shown schematically in (Fig. 4.4) were used. The wild type and mutant expression plasmids were transfected into Vero cells to evaluate the ability of various Vpx mutants to be targeted to the nucleus. Indirect immunofluorescence clearly show Vpx specific signal in all the transfected cells but the localization pattern of mutants varied dramatically

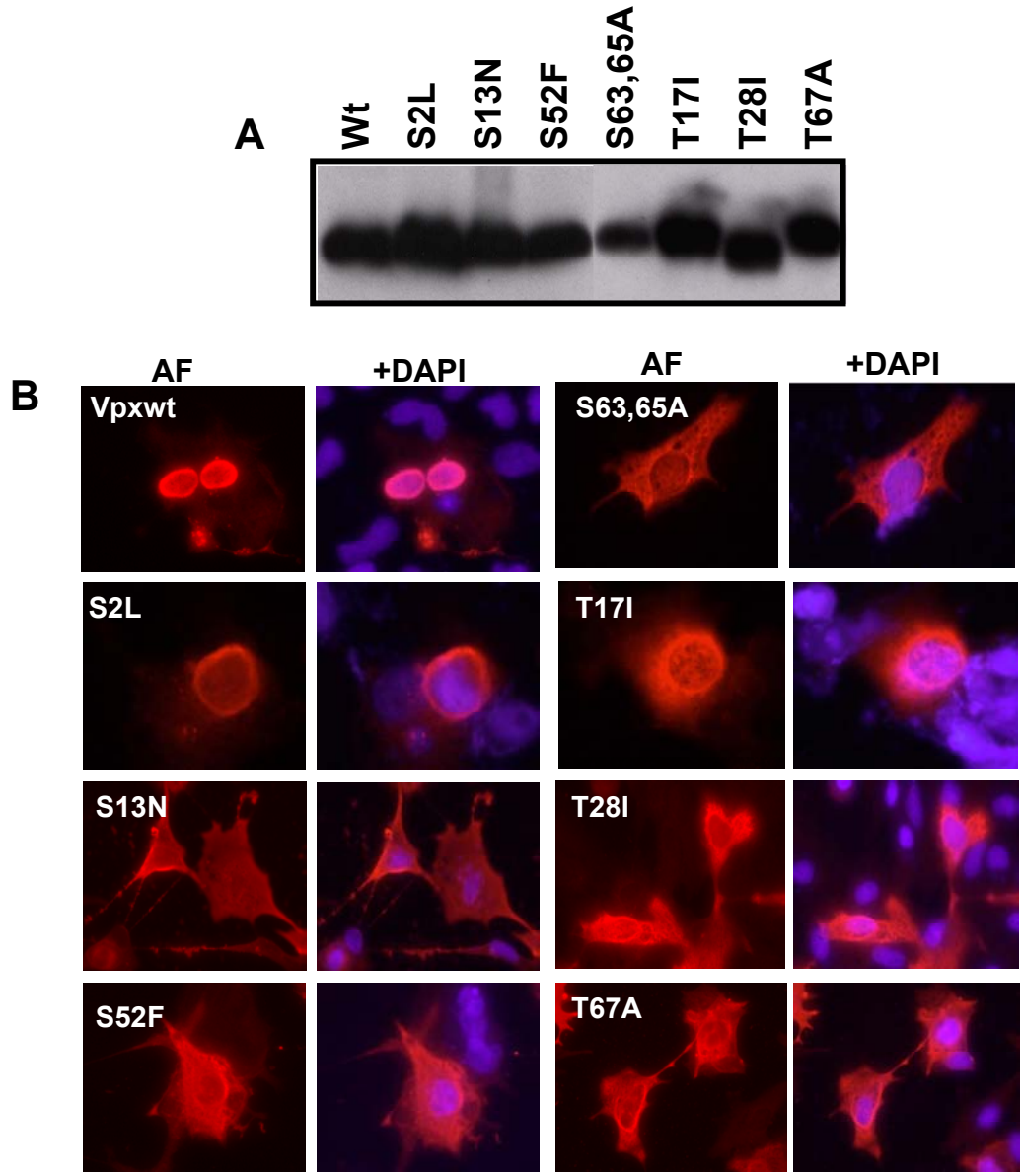


Figure 4.5: Expression and sub-cellular localization of Vpx serine & threonine mutant proteins. A. vTF73 infected Vero cells were transfected with wild type and mutant Vpx expression plasmids. Twelve hours after transfection the cells were harvested and lysed in lysis buffer. The samples were resolved on SDS-12% PAGE followed by Western blot analysis using anti-Vpx monoclonal antibody. **B.** vTF73 infected Vero cells were transfected with wild type and mutant Vpx expression plasmids. Twelve hours after the transfection, localization was detected by indirect immunofluorescence with anti-Vpx monoclonal antibody followed by goat anti-mouse Alexa fluor 594 conjugated secondary antibody. AF: Alexa fluor 594 staining for Vpx (red); DAPI: staining of nuclear compartment (blue)

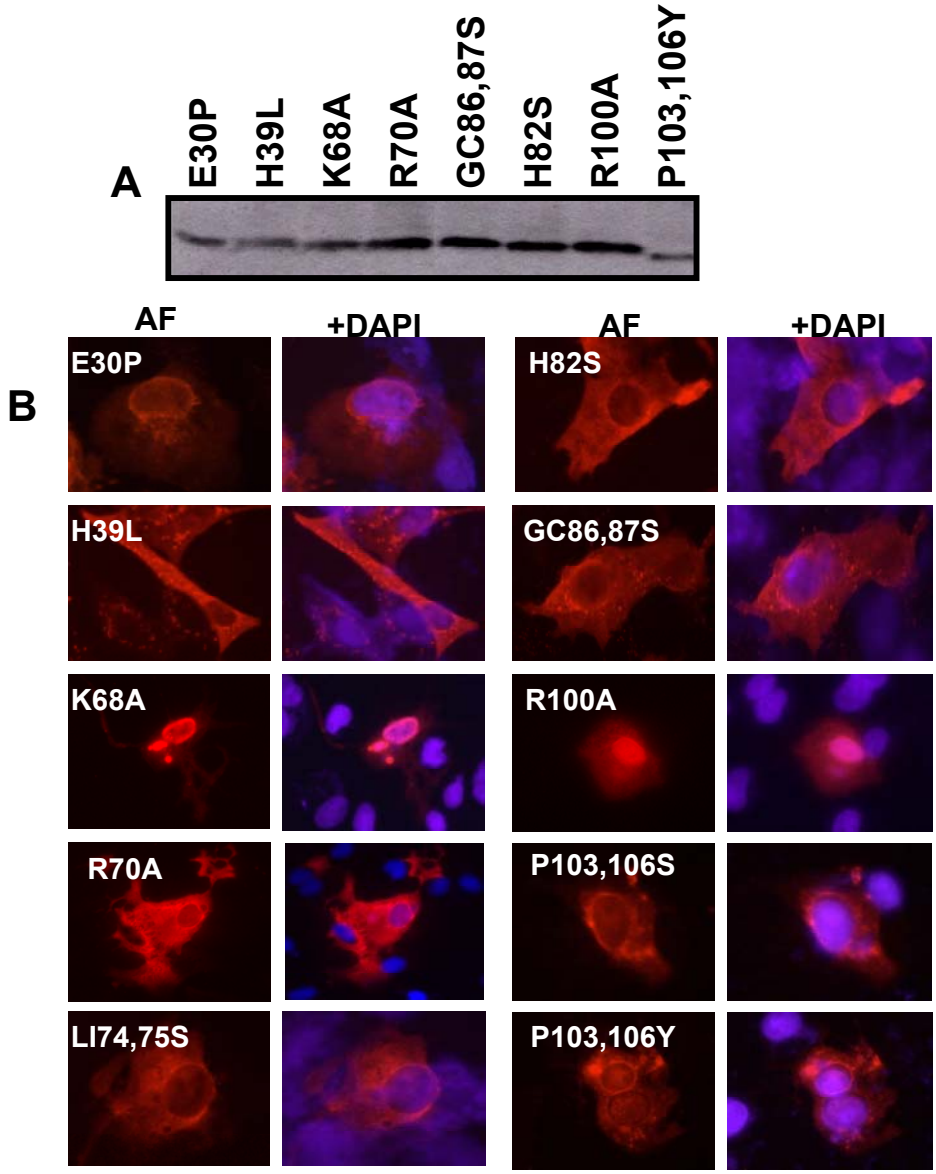


Figure 4.6: Expression and sub-cellular localization of Vpx mutant proteins. A. vTF73 infected Vero cells were transfected with indicated mutant Vpx expression plasmids. Twelve hours after transfection the cells were harvested and lysed in lysis buffer. The samples were resolved on SDS-12% PAGE followed by Western blot analysis using anti-Vpx monoclonal antibody. **B.** vTF73 infected Vero cells were transfected with mutant Vpx expression plasmids. Twelve hours after the transfection, localization was detected by indirect immunofluorescence with anti-Vpx monoclonal antibody followed by goat anti-mouse Alexa fluor 594 conjugated secondary antibody. AF: Alexa fluor 594 staining for Vpx (red); DAPI: staining of nuclear compartment (blue)

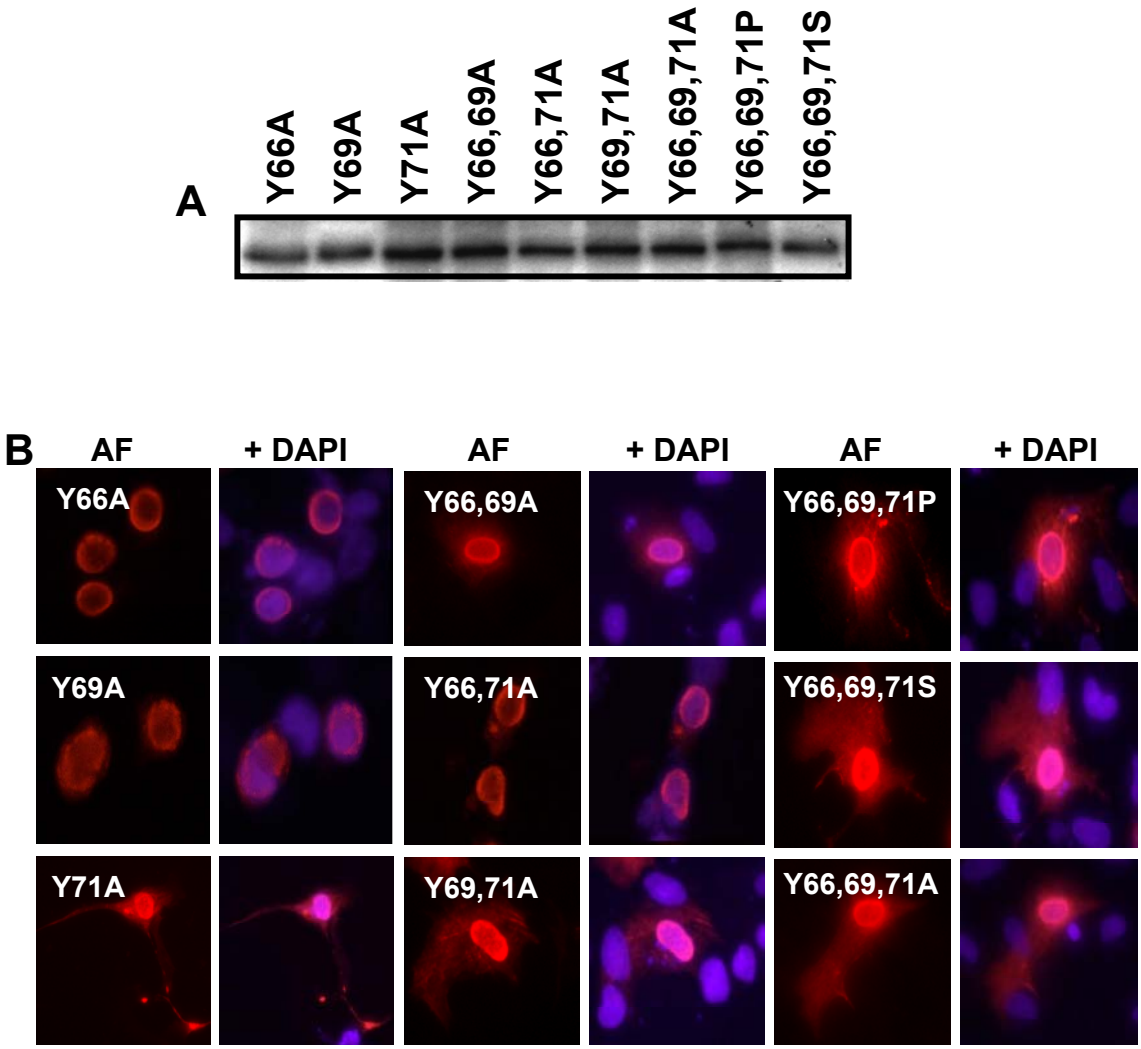


Figure 4.7: Expression and sub-cellular localization of Vpx tyrosine mutant proteins. A. vTF73 infected Vero cells were transfected with Vpx tyrosine mutant expression plasmids. Twelve hours after transfection the cells were harvested and lysed in lysis buffer. The samples were resolved on SDS-12% PAGE followed by Western blot analysis using anti-Vpx monoclonal antibody. **B.** vTF73 infected Vero cells were transfected with indicated mutant Vpx expression plasmids. Twelve hours after the transfection, localization was detected by indirect immunofluorescence with anti-Vpx monoclonal antibody followed by anti-mouse Alexa fluor 594 conjugated secondary antibody. AF: Alexa fluor 594 staining for Vpx (red); DAPI: staining of nuclear compartment (blue)

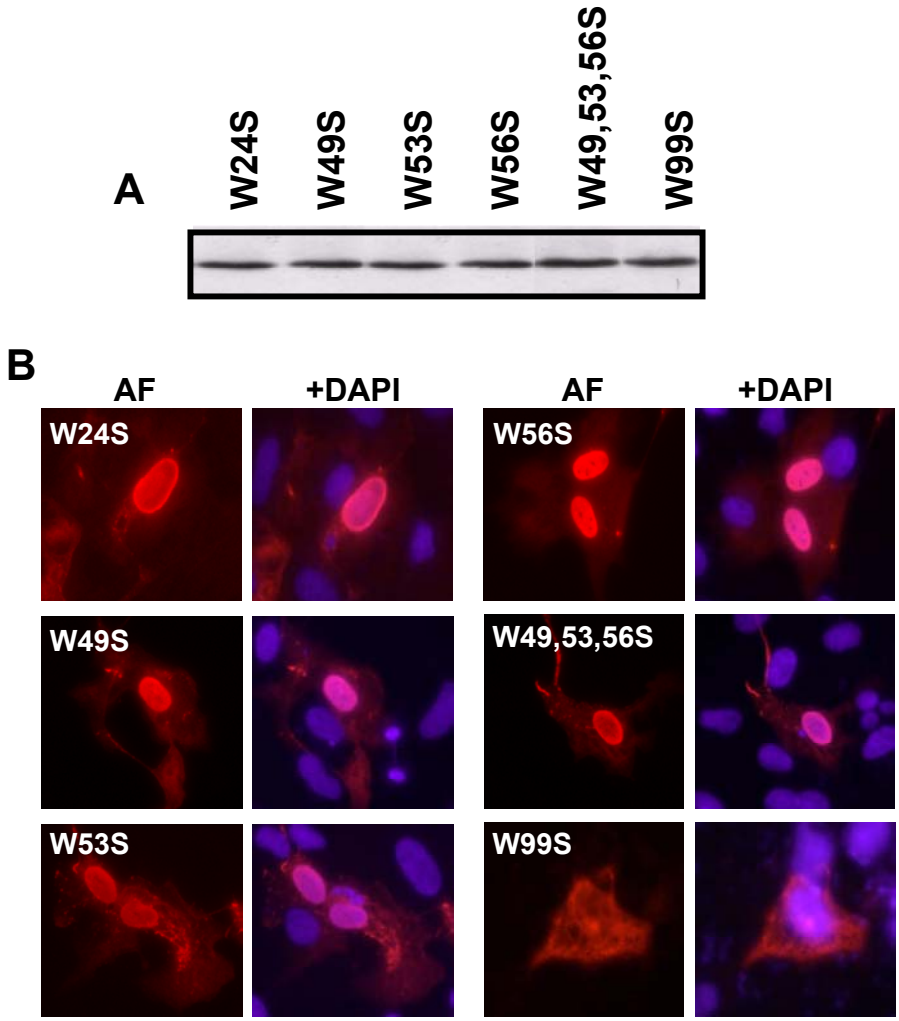


Figure 4.8: Expression and sub-cellular localization of Vpx tryptophan mutant proteins. A. vTF73 infected Vero cells were transfected with Vpx tryptophan mutant expression plasmids. Twelve hours after transfection the cells were harvested and lysed in lysis buffer. The samples were resolved on SDS-12% PAGE followed by Western blot analysis using anti-Vpx monoclonal antibody. **B.** vTF73 infected Vero cells were transfected with wild type and mutant Vpx expression plasmids. Twelve hours after the transfection localization was detected by indirect immunofluorescence with anti-Vpx monoclonal antibody followed by anti-mouse Alexa fluor 594 conjugated secondary antibody. AF: Alexa fluor 594 staining for Vpx (red); DAPI: staining of nuclear compartment (blue)

from wild type protein. Three different patterns of protein localization were observed. Predominantly nuclear like wild-type (E30, S2, Y66, Y69, Y71, W24, W49, W53, W56, and K68); both nuclear and cytoplasmic (S13, T17, T28, T67, S63, S65, R70, G86, C87, W99, R100, P103 and P106); and predominantly cytoplasmic (H39, L74, I75 and H82) (Fig. 4.5B, 4.6B, 4.7B and 4.8B). Similar localization pattern was observed for all the Vpx mutants in three independent experiments. Although there was considerable variation between individual cells in the intensity of fluorescence staining, but the fluorescence intensity and the patterns of sub-cellular localization were not correlated. Taken together, these results strongly suggested that conserved serine and threonine residues as well as the domains in the C-terminus play an important role in Vpx nuclear import. Furthermore the data suggest that mutation in the helical domain III (aa 64-82) impairs Vpx nuclear import suggesting that structural integrity of this helical motif plays an indispensable role in Vpx nuclear transport.

4.5 Vpx nuclear import is a signal mediated process.

Proteins with molecular mass less than 35-40 kDa can enter the nucleus by passive diffusion rather than by a signal-mediated process. To distinguish between these two possibilities, we evaluated the nuclear import activities of Vpx in the context of a chimeric protein designed to exceed the diffusion limit of the nuclear pore. Wild type Vpx protein (18 KDa) was expressed as a fusion protein with 90 kDa β-Galactosidase (β-Gal) and analyzed for nuclear import in Vero cells. We selected β-Gal as a fusion partner because the size of the fusion protein exceeds the passive diffusion limit of nuclear pore complex. Further, β-Gal is known to localize to the nucleus when attached to a functional NLS. β-Gal/Vpx fusion protein accumulated both on the nuclear membrane and inside the nucleus (Fig. 4.10B), by contrast β-Gal accumulated only in the cytoplasm (Fig. 4.10B). This shows that Vpx posses a specific signal that confers nuclear localization.

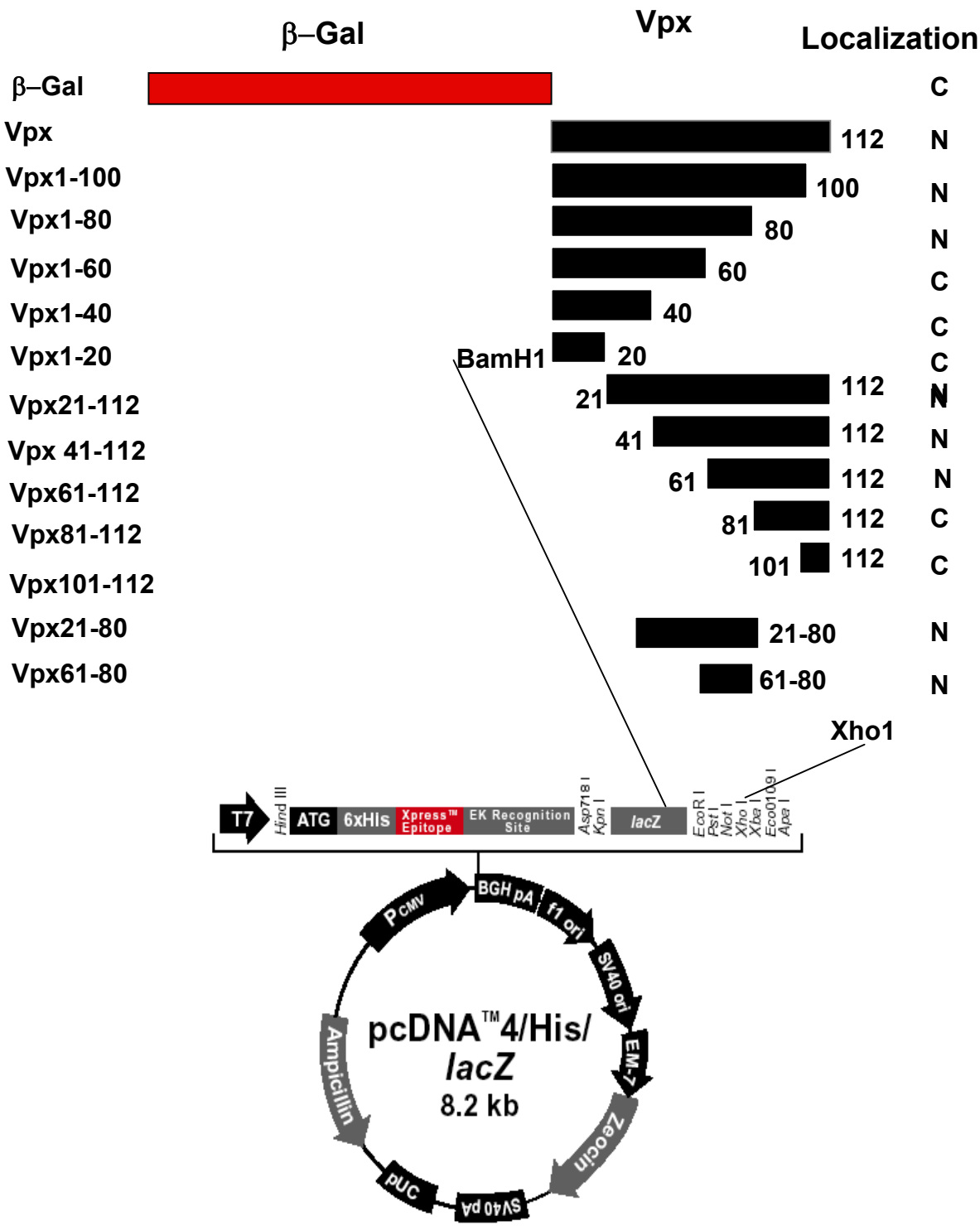


Figure 4.9: Construction of β-Galactosidase-Vpx fusion proteins. Schematic diagram of NH2- and COOH-terminal deletions of Vpx expressed as β-Gal fusion proteins. PCR amplified *vpx* deletion fragments were digested with BamH1(located near the C-terminus of lacZ) and Xho1 and ligated with mammalian expression vector pCDNA4.His Max-β-Gal.

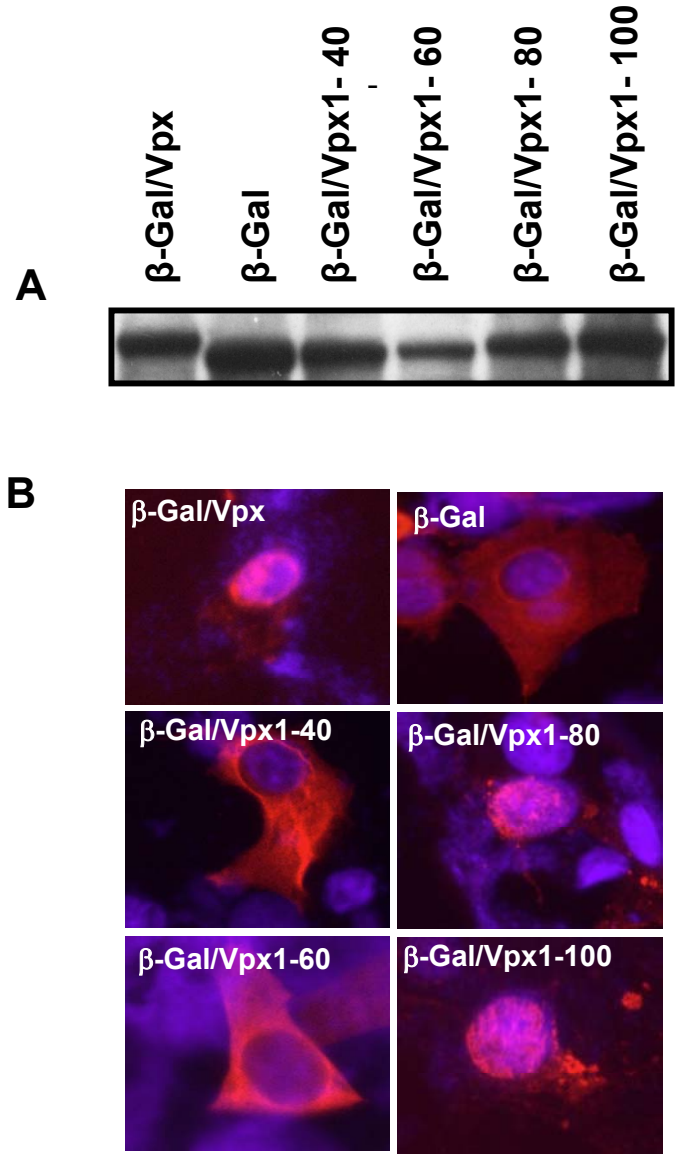


Figure 4.10: Expression and sub-cellular localization of β-Gal/Vpx C-terminal deletions. A. vTF7-3 infected Vero cells were transfected with various β-Gal/Vpx deletion expression plasmids. Twelve hours after transfection the cells were harvested and lysed in lysis buffer. The samples were then resolved on SDS-10% PAGE followed by Western blot analysis using anti- β-Gal monoclonal. All the deletion constructs were found to be of the expected size. **B.** vTF7-3 infected Vero cells were transfected with various β-Gal/Vpx deletion constructs. Localization was detected by indirect immunofluorescence with anti-β Gal monoclonal antibody followed by Alexa fluor 594 conjugated secondary antibody 12h after transfection. Localization of β-Gal Vpx deletions is indicated by red fluorescence (Alexa fluor 594) and staining of the nuclear compartment by DAPI is indicated by blue fluorescence.

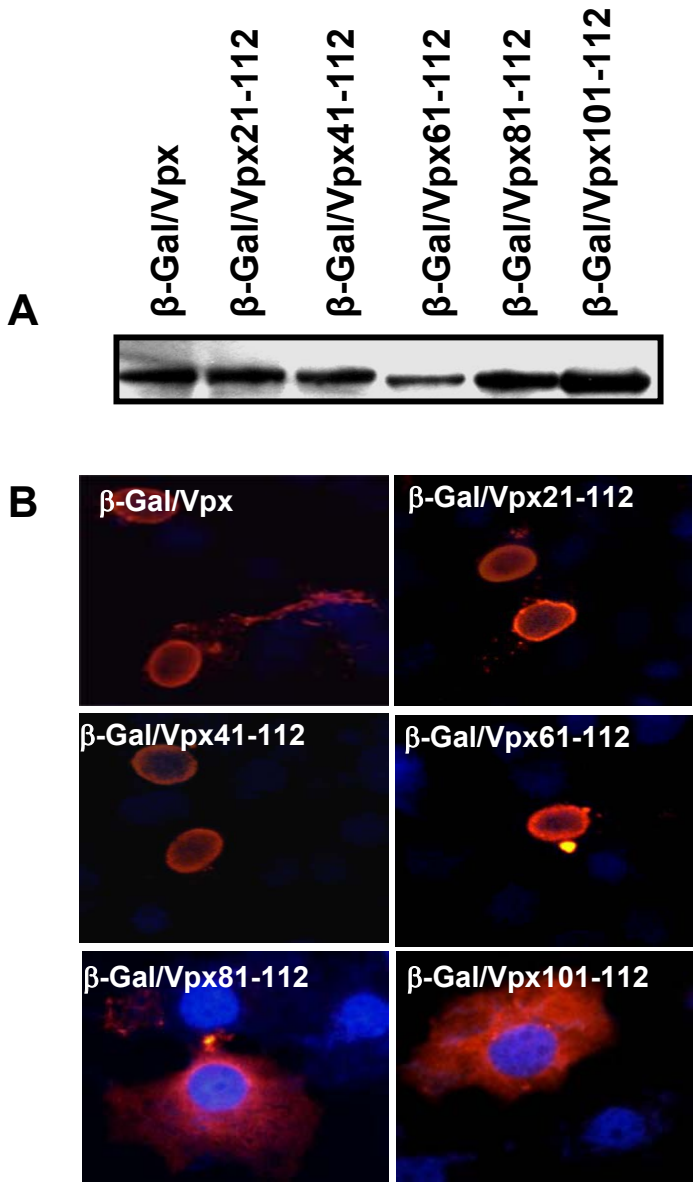


Figure 4.11: Expression and sub-cellular localization of β-Gal/Vpx N-terminal deletions. A. vTF7-3 infected Vero cells were transfected with various β-Gal/Vpx deletion expression plasmids. Twelve hours after transfection the cells were harvested and lysed in lysis buffer. The samples were resolved on a SDS-10% PAGE followed by Western blot analysis using anti-β Gal monoclonal antibody. **B.** vTF7-3 infected Vero cells were transfected with various β-Gal Vpx deletion constructs. Localization was detected by indirect immunofluorescence with anti- β Gal monoclonal antibody followed by Alexa fluor 594 conjugated secondary antibody 12h after transfection. Localization of β-Gal Vpx deletions is indicated by red fluorescence (Alexa fluor 594) and staining of the nuclear compartment by DAPI is indicated by blue fluorescence.

4.6 Identification of the minimal nuclear-targeting signal.

To delineate the sequences that are important for nuclear accumulation of Vpx, NH_2-and COOH-terminal truncations of Vpx were fused with β-Gal (Fig. 4.9). Western blot analysis of whole cell extracts of transfected cells with β-Gal monoclonal antibody confirmed that all the fusion constructs expressed at the right size (Fig. 4.10A and 4.11A). The sub-cellular localization of the chimeric proteins was visualized with confocal as well as immunofluorescence microscopy. Wild type β-Gal protein was seen predominantly in the cytoplasm (Fig. 4.10B) whereas chimeric β-Gal containing full length Vpx was seen predominantly in the nuclear membrane and inside the nucleus (Fig. 4.10B). From the results in figures 4.10B and 4.11B it was quite evident that fusion constructs lacking the amino acid residues 61-80 were primarily localized in the cytoplasm. As shown by confocal microscopy, most of the β-Gal/Vpx 1-80 fusion protein was concentrated on the nuclear membrane and inside the nucleus (Fig. 4.12 A), similar to full length Vpx. Conversely, constructs that lack amino acid residues 61-80 (β-Gal/Vpx 81-112) was localized only in the cytoplasm (Fig. 4.12 B). These results raised the possibility that the domain between amino acid residues 61-80 might contain a novel nuclear import signal. Therefore, to check whether the 20 amino acid stretch spanning 61-80 is necessary and sufficient to transport Vpx into nucleus, we generated a construct containing β-Gal and Vpx amino acids 61-80. As shown in figure 4.13, the resulted β-Gal fusion protein efficiently imported to the nuclear membrane and inside the nucleus, similar to full length Vpx. Furthermore, individual mutations in this region (S63, 65A, R70A and LI 74, 75S) abolished Vpx nuclear import. Collectively, these results suggest that the 20 amino acids (61-80) of Vpx is a novel transferable transport signal (Fig. 4.16) containing the information necessary and sufficient to transport the heterologous cytoplasmic protein to nucleus.

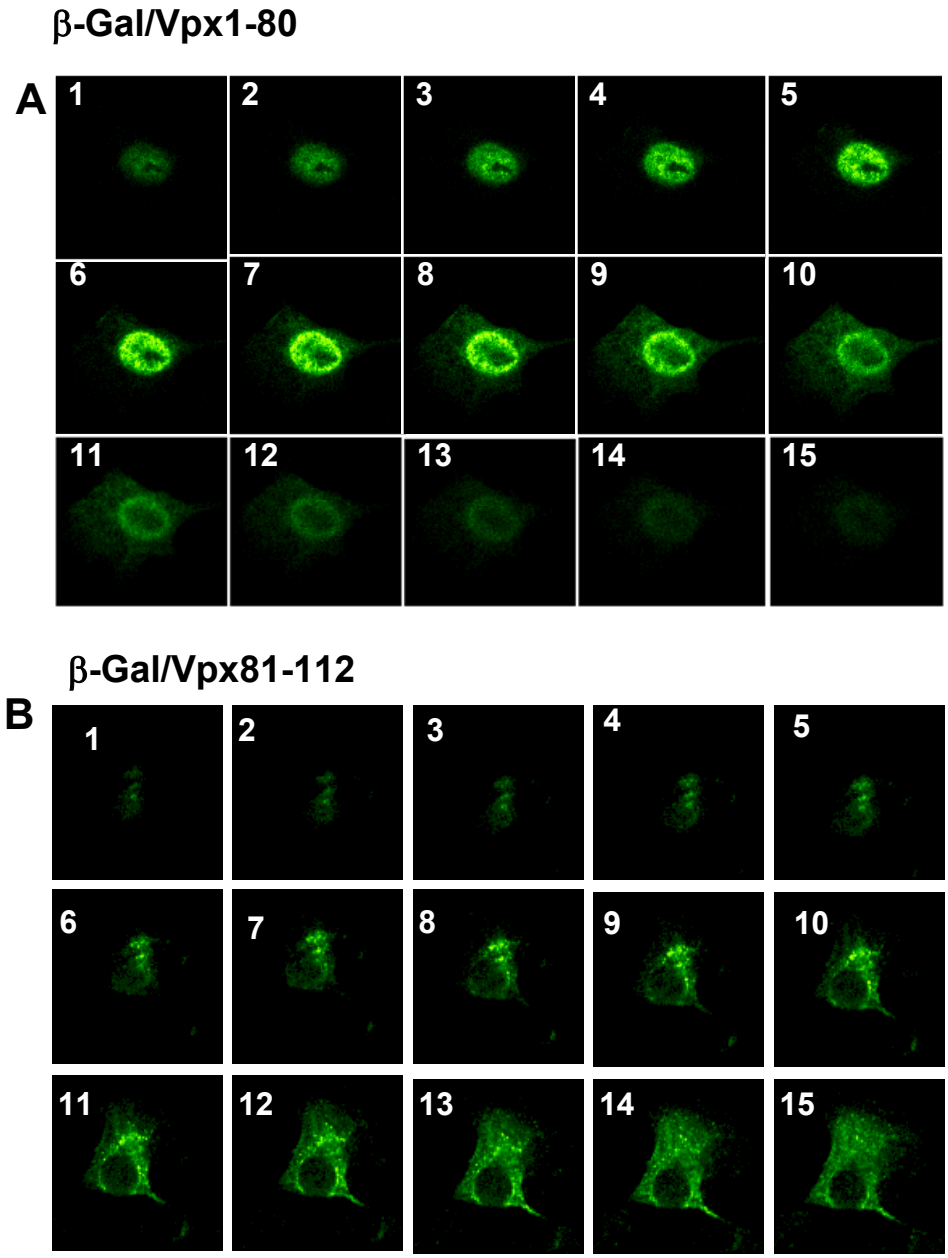


Figure 4.12: Delineation of minimal Vpx nuclear targeting signal. vTF7-3 infected Vero cells were transfected with **A.** β-Gal/Vpx1-80, and **B.** β-Gal/Vpx81-112 expression plasmids. Localization of β-Gal/Vpx fusion proteins were observed by indirect immunofluorescence with an anti β-Gal monoclonal antibody followed by goat anti-mouse FITC. Serial laser sections through a representative cell that expressed β-Gal/Vpx1-80 and β-Gal/Vpx81-112 protein were obtained by confocal laser scanning microscopy at 0.5μm intervals. Photomicrographs were numbered 1, which corresponds to the nuclear periphery, through 15 which corresponds to the region through which the cell was adsorbed to the surface.

β-Gal/Vpx61-80

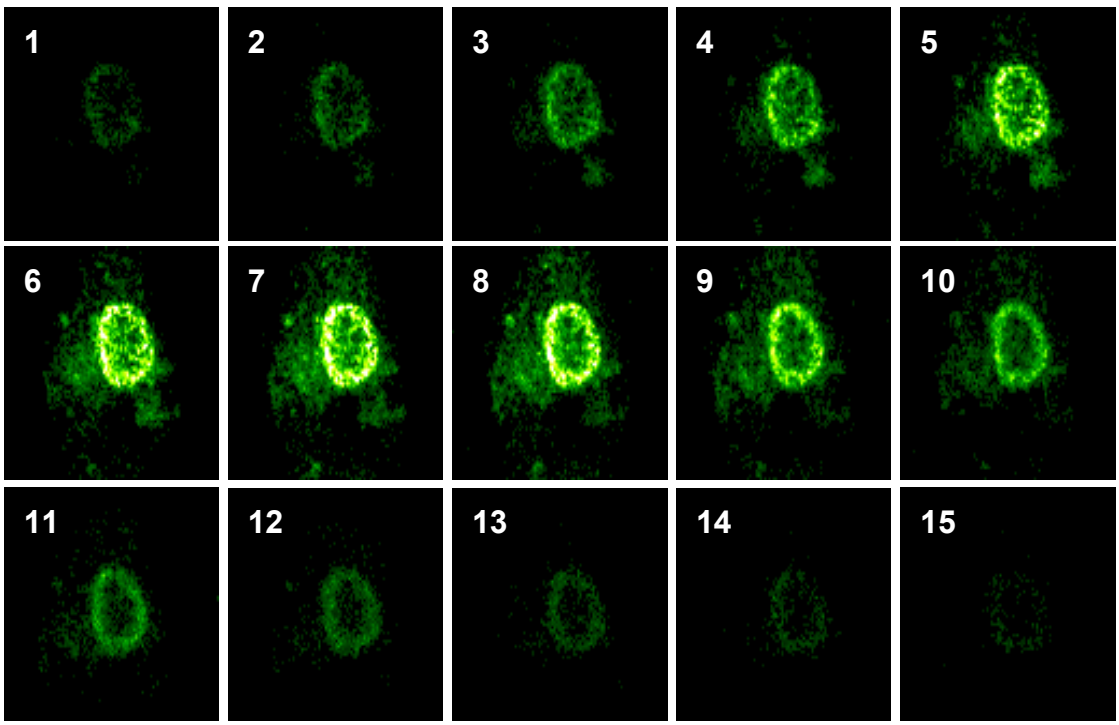


Figure 4.13: Confocal section analysis of Vpx minimal nuclear targeting signal. vTF7-3 infected Vero cells were transfected with β-Gal/Vpx 61-80 expression plasmid. Localization of β-Gal/Vpx fusion protein was observed by indirect immunofluorescence with an anti β-Gal monoclonal antibody followed by anti-mouse FITC. Serial laser sections through a representative cell that expressed β-Gal/Vpx61-80 were obtained by confocal laser scanning microscopy at 0.5μm intervals. Photomicrographs were numbered 1, which corresponds to the nuclear periphery, through 15 which corresponds to the region through which the cell was adsorbed to the surface.

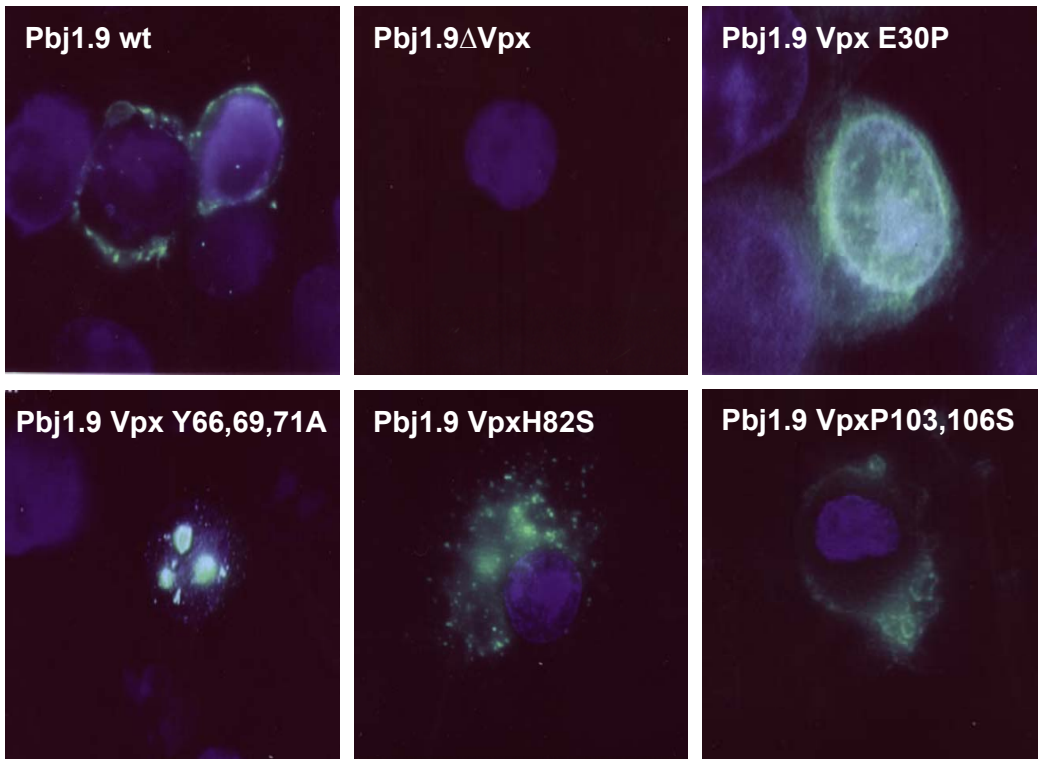


Figure 4.14: Localization of Vpx proteins in SIVsmPBj1.9 infected macaque macrophages. Terminally differentiated macaque macrophage cultures were infected with wild type and Vpx mutant proviruses. Localization of Vpx proteins was detected by indirect immunofluorescence with an anti-Vpx monoclonal antibody followed by goat anti-mouse FITC secondary antibody. Localization of Vpx (green); staining of nuclear compartment with DAPI (blue).

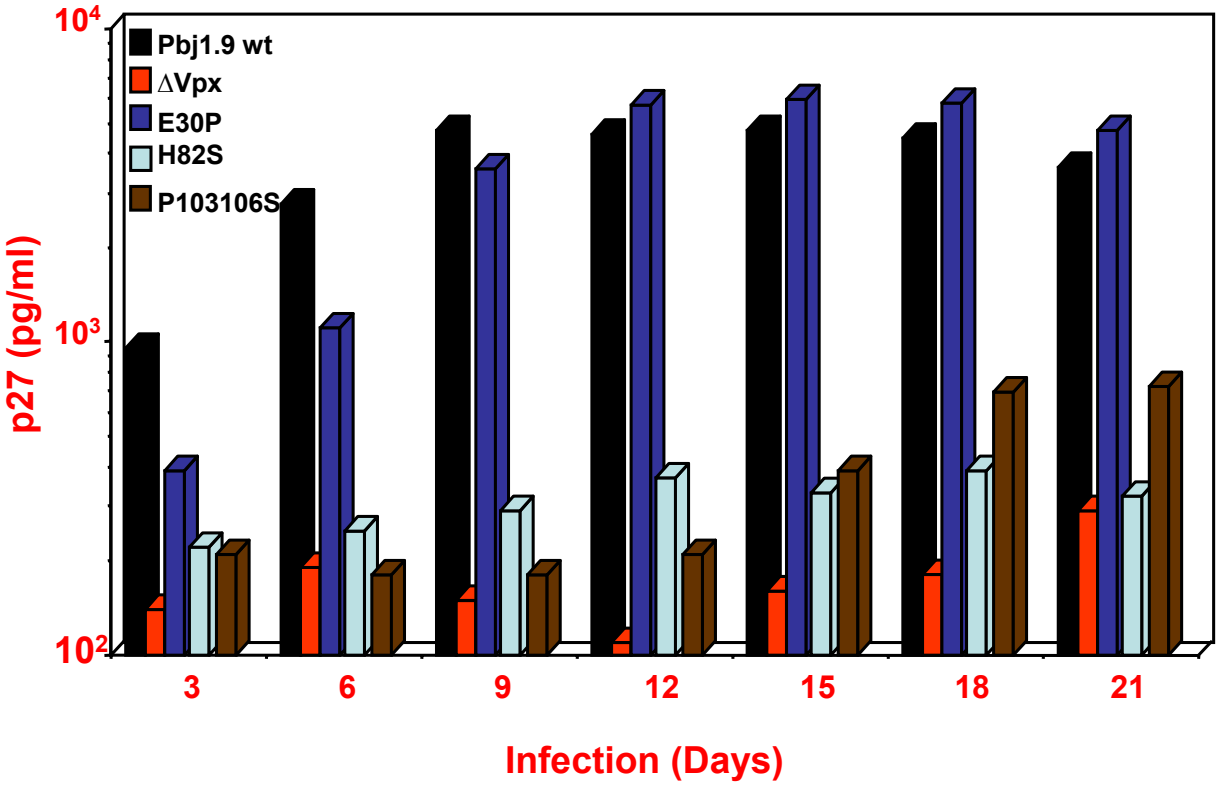


Figure 4.15: Replication kinetics of wild type and *vpx* mutant PBj1.9 proviruses. Terminally differentiated macaque macrophages were infected with equal amount of indicated provirus (equilibrated by p27 Gag content). Virus replication was assessed by quantifying the amounts of p27 Gag antigen in culture supernatants at three day intervals post infection. ΔVpx-control construct lacking functional *vpx* open reading frame.

Name of the signal	Receptors required for transport	Sequence motifs	Examples
cNLS	Importin- α/β	TPPKKKRK VEDP	SV 40 T Antigen
Arginine rich	Importin- α	MPKTRRRPRRS QRKRPPT	HTLV-1 Rex
M9	Transportin	NQSSNFGMKGG NFGGRSSGPYG GGQYFAKPRNQ GGY	hnRNPA1
Vpx NLS	Not known	GM**S**V**S**YT**T**KY**R**YL C**LI**QKAMF	SIVsm Vpx

Figure 4.16: Sequence comparison of various types of NLS. Amino acid sequences of unique NLS of the Vpx protein compared with classical lysine-rich type (SV 40 T antigen NLS), arginine–rich HTLV-1 Rex NLS and M9 signal of hnRNPA1. The residues that are essential for the nuclear transport of Vpx are indicated in bold letters.

4.7 Nuclear import is critical for efficient replication of SIVsmPBj1.9 in macaque macrophages.

Our results were compatible with a role of Vpx in the nuclear import of HIV/SIV PIC and hence for efficient infection in non-dividing cells like macrophages. To probe this issue, we examined the ability of wild-type and nuclear import defective *vpx* mutant SIVsmPBj1.9 proviral constructs to elicit a spreading infection in monocyte-derived macrophage cultures. We selected the proviral clones with three different localization patterns (a, wild-type localization pattern; b, localized to the cytoplasm and c, localized both in the nucleus and cytoplasm) to examine whether nuclear import property of Vpx is required for efficient virus replication in macrophages. Mutant Vpx proteins (H82S, P103,106 S) that failed to localize to the nucleus (Fig. 4.14) were severely impaired in their ability to support virus replication in macrophages (Fig. 4.15). Interestingly the mutant E30P which was designed to disrupt the putative helix I localized to the nucleus (Fig. 4.14) and has shown high levels of replication nearly equal to that of wild type (Fig. 4.15). In three independent experiments, PBj1.9 mutants with substitution in the C-terminal half of the Vpx replicated poorly in macrophages. Taken together, these data suggest that the nuclear import function of Vpx is necessary for optimal virus replication in non-dividing cells such as macrophages.

Chapter Five

Phosphorylation regulates Vpx nuclear transport

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Phosphorylation plays a critical role in the nuclear localization signal mediated nuclear transport, cell cycle progression, and gene expression. Phosphorylation-regulated NLSs were found to control nuclear transport in eukaryotic cells from yeast and plants to higher mammals. Studies in HIV-1 have shown that phosphorylation of various viral proteins play a critical role in viral infectivity and replication. Proteins like Gag p17 MA, Gag p24 CA, Vif, Vpu, Rev, and Nef were shown to be phosphorylated by cellular kinases both *in vitro* and *in vivo* (Burnette *et al*., 1993; Hauber *et al*., 1988; Schubert *et al*., 1992; Yang *et al*., 1996; Yang and Gabzuda, 1998). Recent studies have demonstrated that serine-threonine kinases of the host cell are incorporated into HIV-1 particles and regulate early steps in life cycle (Jacque *et al*., 1998). In HIV-1, virion associated cAMP dependent protein kinase A phosphorylates p24 CA and regulates virus particles release from the infected cells (Cartier *et al*., 2003). Similarly phosphorylation of Gag p17MA and p6 by the virion associated MAPK/ERK-2 was shown to be essential for p17MA association with the viral PIC, regulating viral infectivity (Kaushik and Ratner, 2004; Camaur *et al*., 1997; Hemonnot *et al*., 2004). Vpx was shown to be the major determinant for efficient HIV-2/SIVsm replication in non-dividing cells such as macrophages but the mechanism that governs Vpx nuclear import was not known. This part of the study was designed to elucidate the mechanism(s) regulating Vpx nuclear import and its effect on viral replication in target cells like macrophages.

5.1 Vpx is phosphorylated by cellular kinases.

In order to define whether phosphorylation regulates Vpx functions, we first checked the phosphorylation status of Vpx. Vpx expression plasmid was transfected into Vero cells following the infection with vTF7-3 as described in Materials and Methods (section 3.7). After 10-12 hours of transfection, cells were labeled with phosphate free DMEM containing ^{32}P orthophosphoric acid (1 mCi/ml) or with 0.3 mCi of ^{35}S methionine and cysteine in –met/cys DMEM

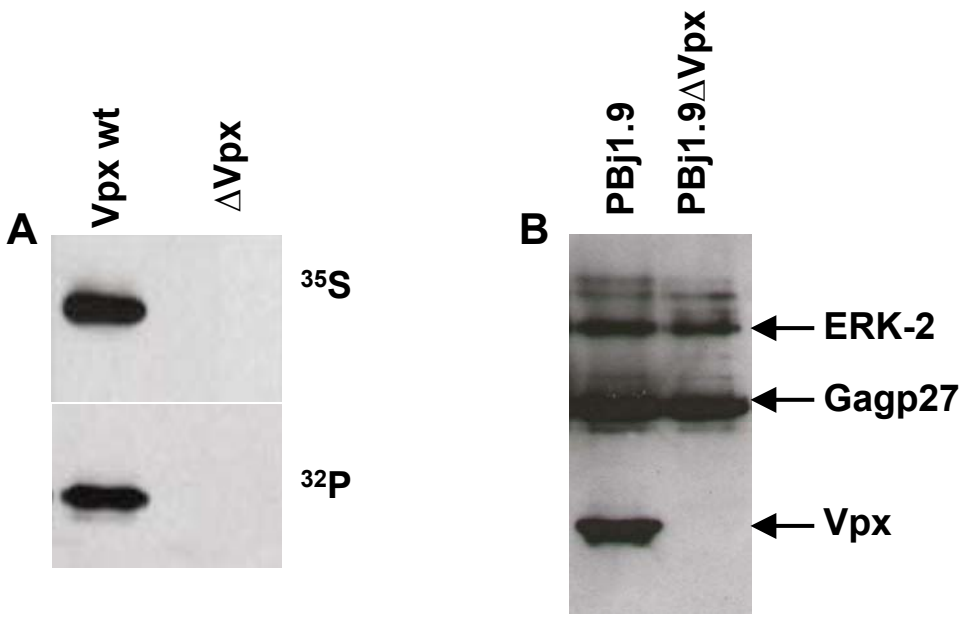

Figure 5.1: Vpx is phosphorylated by cellular kinases. A. vTF7-3 infected Vero cells were transfected with Vpx expression plasmid and labeled with ^{32}P orthophosphoric acid (1mci/ml) in phosphate free DMEM medium containing 1% dialyzed fetal bovine serum or Met/Cys-free DMEM containing 0.3mCi of ^{35} S methionine and cysteine. The labeled cells were lysed and immunoprecipitated with anti-Vpx monoclonal antibody. Proteins were resolved on SDS-15% PAGE followed by autoradiography. **B.** MAPK/ERK-2 is associated with SIVsmPBj1.9 virions. Virus particles were purified from productively infected CEMx174 cells and analyzed by Western blot analysis with anti-ERK-2, anti-Gag and anti-Vpx antibodies. MAPK/ERK-2 is incorporated into the virus particles in the presence or absence of Vpx.

for 6hrs. The labeled cells were lysed and immunoprecipitated with anti-Vpx monoclonal antibody and separated on a SDS-15% PAGE. A ^{32}P-labeled band was visible in the cells expressing Vpx but not in the cells transfected with *Δvpx*, suggesting that Vpx is phosphorylated by cellular kinases (Fig. 5.1A).

Previous studies suggested that cellular kinases like MAPK/ERk-2 and cAMP dependent protein kinase A are selectively incorporated into HIV-1 particles and regulate virus infectivity (Cartier *et al*., 2003; Cartier *et al*., 1997). We therefore first investigated for the presence of cellular kinases in purified SIVsmPBj1.9 virions. Viral supernatants were clarified from productively infected CEMx174 cell lines and purified through a 20% sucrose cushion. The purified virions were then analyzed by Western blot with antibodies against MAPK/ERK-2, Gag p27, and Vpx. The results in figure 5.1B indicate that MAPK/ERK-2 was selectively incorporated into the virus particles irrespective of Vpx incorporation.

5.2 MAPK/ERK-2 phosphorylates Vpx both *in vivo* and *in vitro.*

We next examined whether Vpx is a substrate for MAPK/ERK-2 and stimulation or inhibition of the MAPK pathway modulates the phosphorylation of Vpx. The mitogen phorbol myristate acetate (PMA) was used to activate the MAPK pathway in Jurkat cells. Direct evidence of Vpx phosphorylation was obtained by immunoprecipitation of various cellular kinases (MAPK/ERK-2, MAPK/ERK-1, NIK, LCK) from PMA activated Jurkat cell lysates, followed by *in vitro* kinase assay using recombinant Vpx protein. The kinase assay product was resolved on a SDS-15% PAGE followed by autoradiography. The results in figure 5.2A provide evidence that Vpx is specifically phosphorylated by MAPK/ERK-2 but not by other kinases.

Since ERK-2 dependent phosphorylation was reported for Gagp17MA, we used GST and GST-Gag p17MA as negative and positive controls,

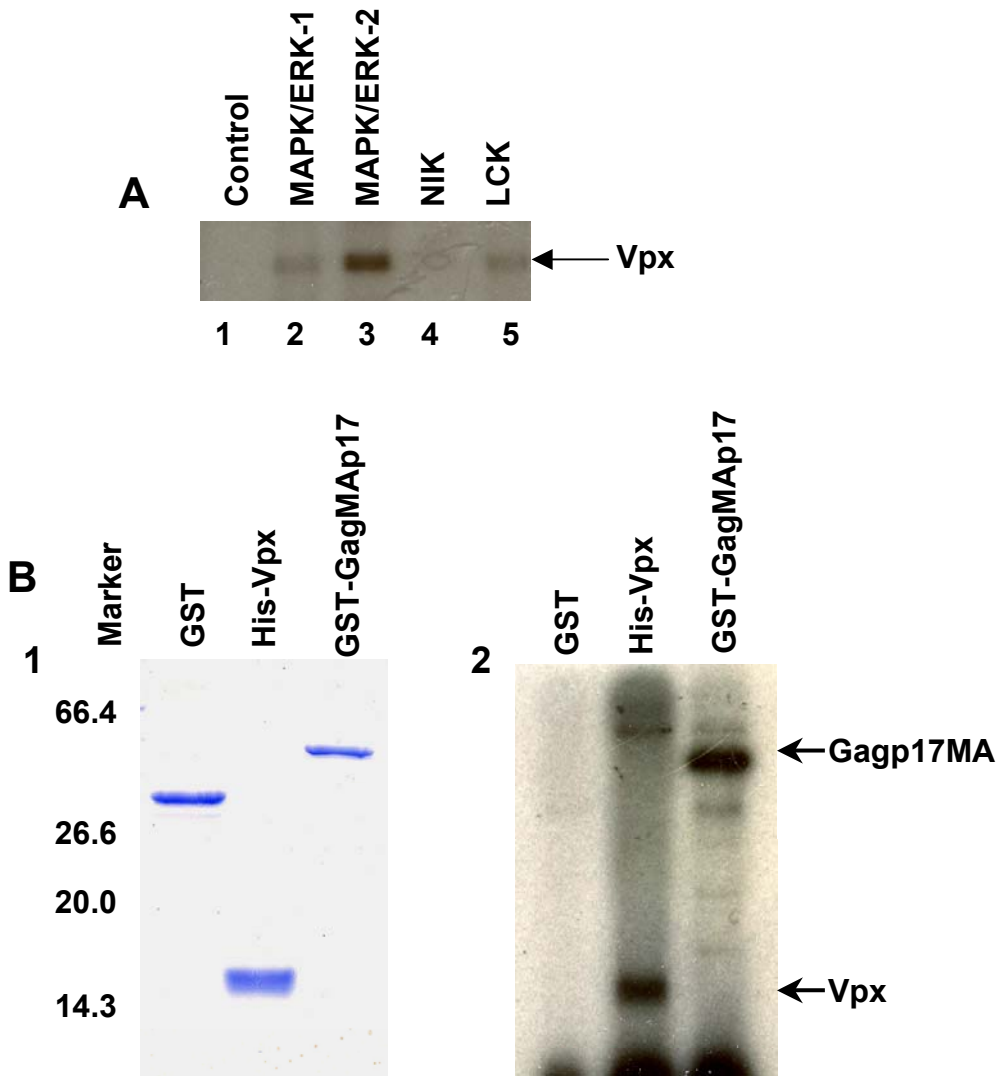

Figure 5.2: Vpx is phosphorylated by MAPK/ERK-2. A. Various cellular kinases were immunoaffinity purified from PMA–activated Jurkat cells, and the activity was examined by *in vitro* kinase assay using purified recombinant SIVsm Vpx. Phosphorylated Vpx protein was resolved on SDS-15% PAGE followed by autoradiography. Vpx was phosphorylated specifically by ERK2 but not by other kinases. **B.** Recombinant MAPK/ERK-2 phosphorylates Vpx. 1). Vpx, GST and GST-GAGp17MA were expressed in *E.coli,* BL21-DE3 and purified by nickel-nitrilo triacetic acid affinity chromatography (for Vpx) and glutathione sepharose (for GST and GST-Gagp17MA). The proteins were separated on SDS-15% PAGE followed by staining with coomasie brilliant blue. 2). Purified recombinant GST, Vpx and GST-Gag p17MA were used as substrates for an *in vitro* kinase assay. Vpx and GST-Gag p17MA were phosphorylated by recombinant ERK-2.

respectively in the *in vitro* kinase assay. Recombinant MAPK/ERK-2 was able to phosphorylate recombinant Vpx and Gag p17MA (Fig. 5.2. B2). An equal amount of protein was used for kinase reaction as ascertained by coomasie brilliant blue staining (Fig. 5.2. B1). The results clearly indicate that MAPK/ERK-2 phosphorylates Vpx both *in vivo* as well as *in vitro.*

5.3 MAPK/ERK-2 inhibitor Hypericin prevents phosphorylation of Vpx.

Hypericin was known to inhibit the activity of MAPK/ERK2, so to further understand the specificity of Vpx phosphorylation by the host cellular kinase MAPK/ERK-2, we analyzed whether MAPK/ERK-2 mediated phosphorylation of Vpx is inhibited by hypericin. MAPK/ERK-2 was immunoaffinity purified from PMA activated Jurkat cells in the presence or absence of hypericin, and kinase activity was determined by using a recombinant Vpx as a substrate. The results in figure 5.3A suggest that hypericin inhibited MAPK/ERK-2 mediated phosphorylation of Vpx. Western blot analyses of cell lysates from Jurkat cells treated with hypericin indicated that hypericin has inhibited only the activity of MAPK but has not altered the level of its expression (Fig. 5.3A; Lower panel).

We next analyzed whether hypericin selectively inhibits ERK-2 activity but not the activity of other serine-threonine kinases. ERK-2 and JNK (another serine-threonine kinase in the MAPK pathway) were immunoaffinity purified from Jurkat cells treated with or without 100 nm/ml of hypericin and subjected to *in vitro* kinase reaction using Vpx and c-Jun as substrates respectively. The results in figure 5.3B indicate that Vpx is selectively phosphorylated by ERK-2. Complete inhibition of ERK-2 mediated Vpx phosphorylation was noticed in the presence of hypericin. In contrast, equal level of c-Jun phosphorylation by JNK was observed with or without hypericin. Collectively, these results suggest that Vpx is phosphorylated by MAPK/ERK-2 and hypericin selectively inhibits ERK-2 activity.

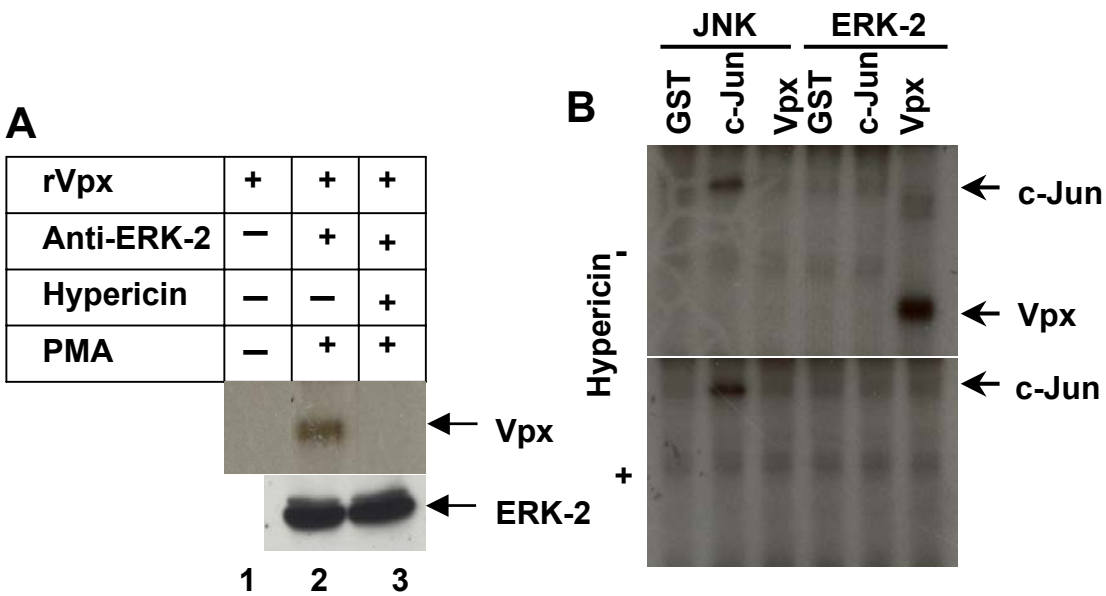

Figure 5.3: MAPK inhibitor hypericin prevent Vpx phosphorylation. A. MAPK/ERK-2 was immunoaffinity purified from PMA activated Jurkat cells in the presence or absence of MAPK inhibitor hypericin, and kinase activity was determined by using recombinant Vpx as a substrate. Hypericin inhibits MAPK/ERK-2 mediated Vpx phosphorylation (lane 3). Western Blot analysis of cell lysates with anti-ERK-2 antibody showed the presence of equal amount of the kinase in both hypericin treated and untreated Jurkat cells (lane 2 and 3, lower panel). **B.** Hypericin specifically inhibits ERK-2 mediated phosphorylation of Vpx. JNK and MAPK/ERK-2 were immunoaffinity purified from Jurkat cells treated with MAPK inhibitor, hypericin (100 nm/ml) and the activity was determined by *in vitro* kinase assay using recombinant c-JUN as a substrate for JNK and Vpx for MAPK/ERK-2.

5.4 Phosphorylation by MAPK/ERK-2 regulates Vpx nuclear transport.

Earlier studies suggest that phosphorylation play a critical role in regulating nuclear transport of various cellular and viral proteins (Fridell et al., 1997; Jans and Hubner 1996; Peterson and Schreiber, 1999). Interestingly, exchange of potential phosphorylation residues like serines and threonines impaired Vpx nuclear transport (Fig. 4.5 B) suggesting that Vpx nuclear import may also be regulated by phosphorylation. Since Vpx is specifically phosphorylated by MAPK/ERK-2, we checked whether MAPK/ERK-2 mediated phosphorylation regulates Vpx nuclear transport. To study this issue in detail, we transfected the vTF7-3 infected cells with Vpx expression plasmid and were labeled with 1mCi of ^{32}P orthophosphoric acid. The presence of phosphorylated form of Vpx in nuclear and cytoplasmic compartments was analyzed as described in Materials and Methods (section 3.9 and 3.13). The results in figure 5.4A show the accumulation of Vpx in the nuclear fraction (N) in the absence of hypericin. In contrast, more Vpx protein was found in cytoplasmic fraction (C), when transfected cells were treated with hypericin (Fig. 5.4A). Interestingly, nearly equal levels of Vpx phosphorylation was observed in the presence or absence of hypericin (Fig. 5.4A), suggesting that Vpx may be phosphorylated at multiple residues and ERK-2 mediated phosphorylation may be required for Vpx nuclear import.

Furthermore indirect immunofluorescence analysis indicated that Vpx localizes predominantly to the nucleus in Vero cells in the absence of any inhibitors. But in the presence of hypericin or PD98059 (an inhibitor of the upstream MEK in the MAPK pathway) Vpx localized to cytoplasm (Fig. 5.4 B). This inhibition of nuclear import was specific, as the tyrosine kinase inhibitor (PP2) 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-*d*] pyrimidine did not affect the nuclear import of both wild type (Fig. 5.5A) as well as Vpx mutant

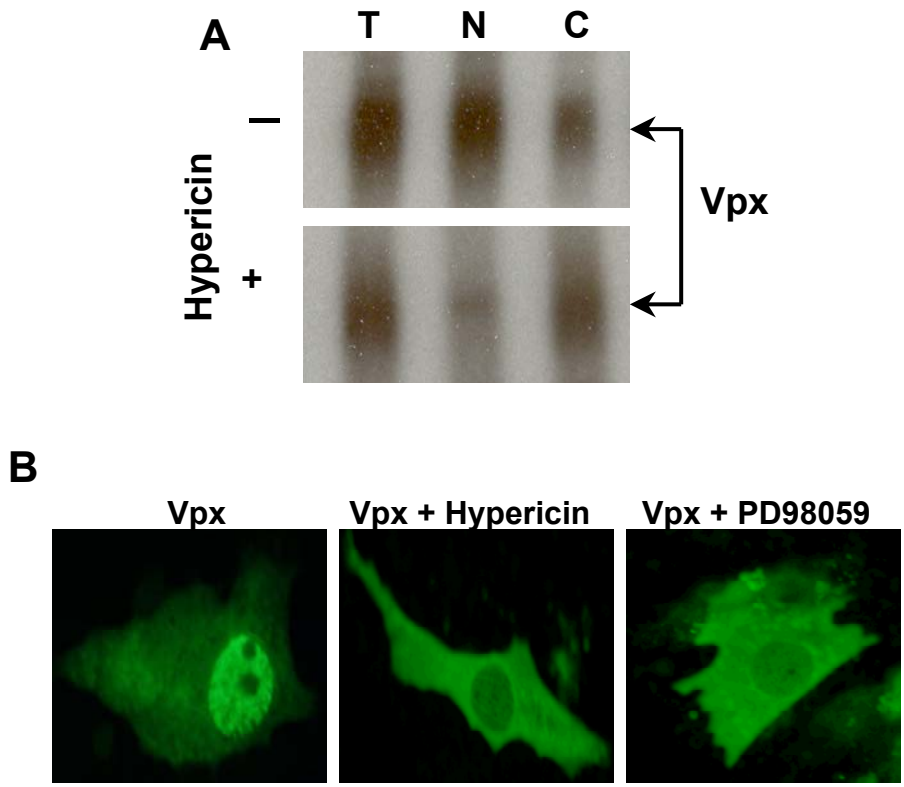

Figure 5.4: MAPK mediated phosphorylation regulates SIVsmPBj1.9 Vpx protein nuclear transport. **A.** vTF7-3 infected Vero cells were transfected with Vpx expression plasmids and labeled with 1 mCi of ^{32}P orthophosphoric acid. The presence of phosphorylated form of Vpx protein in the total cell lysate (T) cytoplasmic (C) and nuclear (N) fractions was determined by immunoprecipitation using monoclonal Vpx antibody after separating the nuclear and cytoplasmic fractions as described in Materials and Methods. **B.** vTF7-3 infected Vero cells were transfected with Vpx expression plasmid. Twelve hours after the transfection the cells were treated with the indicated MAPK pathway inhibitors for 3 hrs. The localization of Vpx was detected by indirect immunofluorescence using anti-Vpx monoclonal antibody followed by anti-mouse Alexa fluor 488-conjugated secondary antibody.

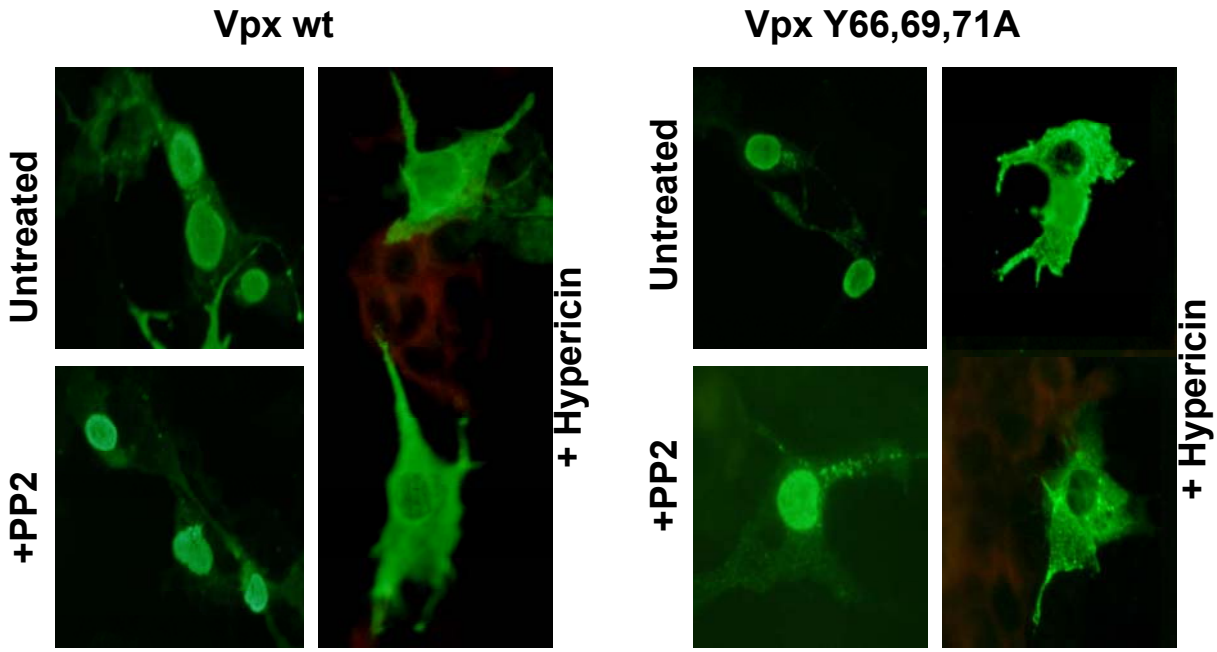

Figure 5.5: MAPK/ERK-2 inhibitor, hypericin alter the nuclear import of Vpx. vTF7-3 infected Vero cells were transfected with Vpx wild type and Y66,69,71A expression plasmids. Twelve hours after transfection the cells were treated with hypericin (100nm) and tyrosine kinase inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7(*t*-butyl)pyrazolo[3-4-*d*]pyrimidine; 5nm) for 3h. The localization of Vpx proteins was visualized by indirect immunofluorescence using monoclonal Vpx antibody followed by anti-mouse Alexa fluor 488 conjugated secondary antibody.

Y66,69,71 (Fig. 5.5B). Collectively, these results suggest that phosphorylation by MAPK/ERK-2 plays an important role in Vpx nuclear transport.

To determine the amino acid residues that are required for Vpx phosphorylation, we transfected vTF7-3 infected Cos-7 cells with various Vpx mutant expression plasmids and labeled with ^{32}P orthophosphoric acid as described in Materials and Methods (section 3.13). Results in figure 5.6 clearly indicate that replacement of serine 63 and 65 has severely impaired phosphorylation suggesting that these amino acid residues are critical for MAPK/ERK-2 mediated Vpx phosphorylation. Similarly reduction in phosphorylation was also observed for L74, I75S, H82S and P103,106S mutants (Fig. 5.7A). But no reduction in phosphorylation was observed when tyrosine and tryptophan residues were exchanged (Fig. 5.6B and 5.7B). Nevertheless an equal amount of expression was seen in all the Vpx mutants (Fig. 5.6 A, B and 5.7 A, B lower panel). To determine the interrelationship between phosphorylation and Vpx nuclear transport, we compared the sub-cellular localization patterns with phosphorylation status of various Vpx mutants. As shown in table 5.1 mutants like S63A/S65A, LI74,75S, H82S, P103,106S, which have shown a reduction in phosphorylation when compared to Vpx wild type were failed to localize to nucleus. Whereas mutants like E30P, Y66A, Y69A, Y71A, Y66,69,71A, K68A which retained wild type levels of phosphorylation localized to nucleus. Collectively, these results indicate that phosphorylation by MAPK/ERK-2 regulates Vpx nuclear transport.

5.5 MAPK/ERK-2 regulates SIVsmPBj1.9 infectivity.

Our results suggested that MAPK/ERK-2 phosphorylates Vpx and regulates its nuclear transport. We next determined whether MAPK/ERK2 mediated phosphorylation of Vpx modulates SIVsmPBj1.9 infectivity. To study this aspect in detail, SIVsmPBj1.9 wild type and SIVsmPBj1.9 Δ *vpx* proviral clones were transfected into 293-T cells. Virus particles were generated in the

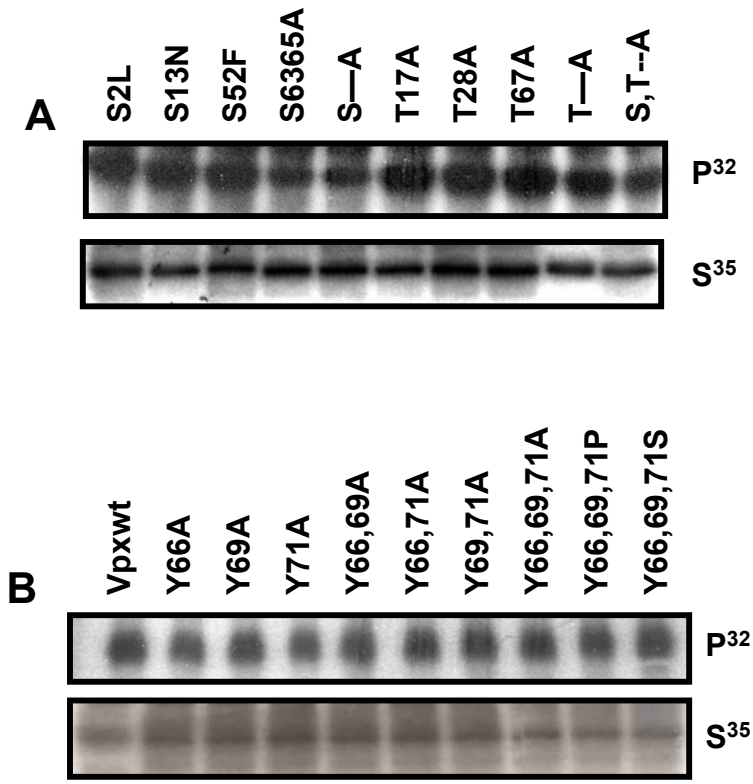

Figure 5.6: Phosphorylation status of Vpx serine, threonine and tyrosine mutant proteins. vTF7-3 infected Cos-7 cells were transfected with indicated Vpx mutant expression plasmids. Twelve hours after transfection the cells were labeled with 1mCi of ^{32}P orthophosphoric acid or 0.3mCi of ^{35}S methionine and cysteine for 6h. Labeled cells were lysed and immunoprecipitated with anti-Vpx monoclonal antibody. Labeled Vpx proteins were separated on SDS-15% PAGE followed by autoradiography. (**A**) S-A, all the serines changed to alanine; T-A all the threonines changed to alanine; S,T-A all serines and threonines changed to alanine. (**B**) Tyrosine mutants did not show any difference in phosphorylation levels when compared with Vpx wt. Nevertheless all the mutant proteins are expressed at equal levels as visualized by ^{35}S labeling.

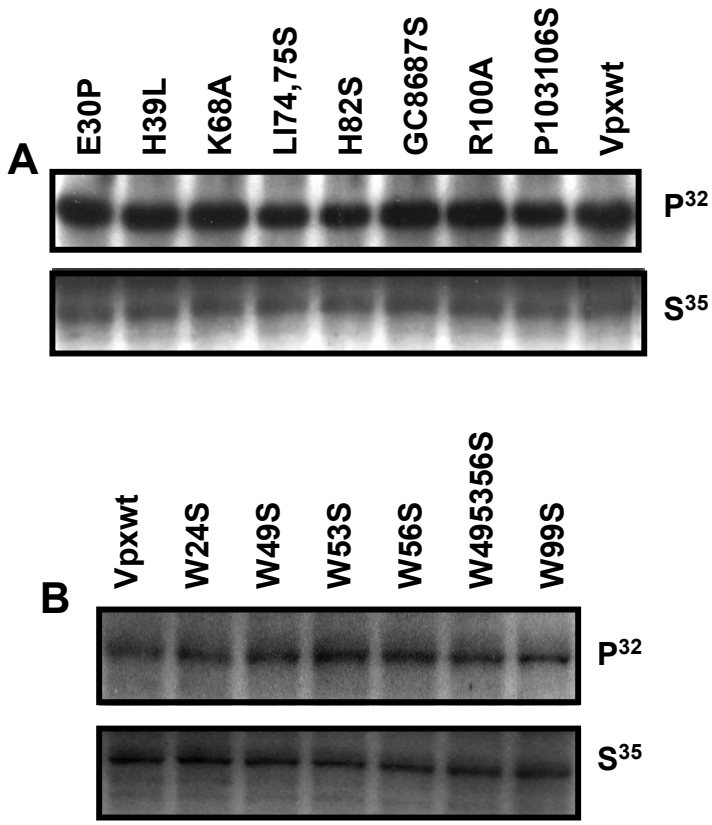

Figure 5.7: Phosphorylation status of Vpx mutant proteins. vTF7-3 infected Cos-7 cells were transfected with indicated Vpx mutant expression plasmids. Twelve hours after transfection the cells were labeled with 1mCi of ^{32}P orthophosphoric acid or 0.3 mCi of ^{35}S methionine and cysteine for 6h. Labeled cells were lysed and immunoprecipitated with anti-Vpx antibody. Labeled Vpx proteins were separated by electrophoresis on SDS-15% PAGE followed by autoradiography. (**A**). Vpx containing mutations in the conserved residues. (**B**) Phosphorylation status of tryptophan mutants.

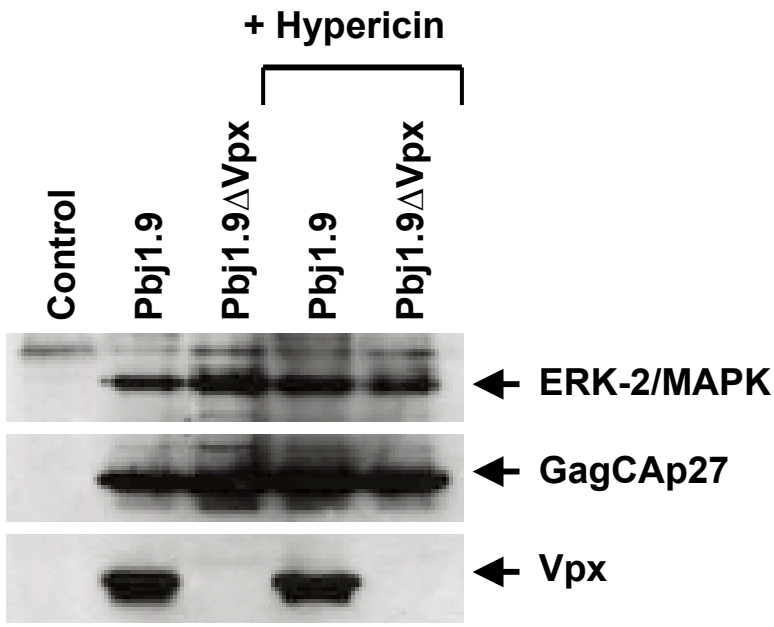

Figure 5.8: MAPK inhibitor hypericin does not alter the packaging of MAPK/ERK-2 into virus particles: Virus particles were obtained from productively infected CEMx174 cells in the presence or absence of MAPK inhibitor, hypericin. The virus particles were purified by collecting the culture supernatants and then were concentrated by ultracentrifugation through a 20% sucrose cushion. Viral pellets were resolved by SDS-12% PAGE, followed by Western Blot analysis using anti-ERK2, anti-Vpx and anti-Gag antibodies.

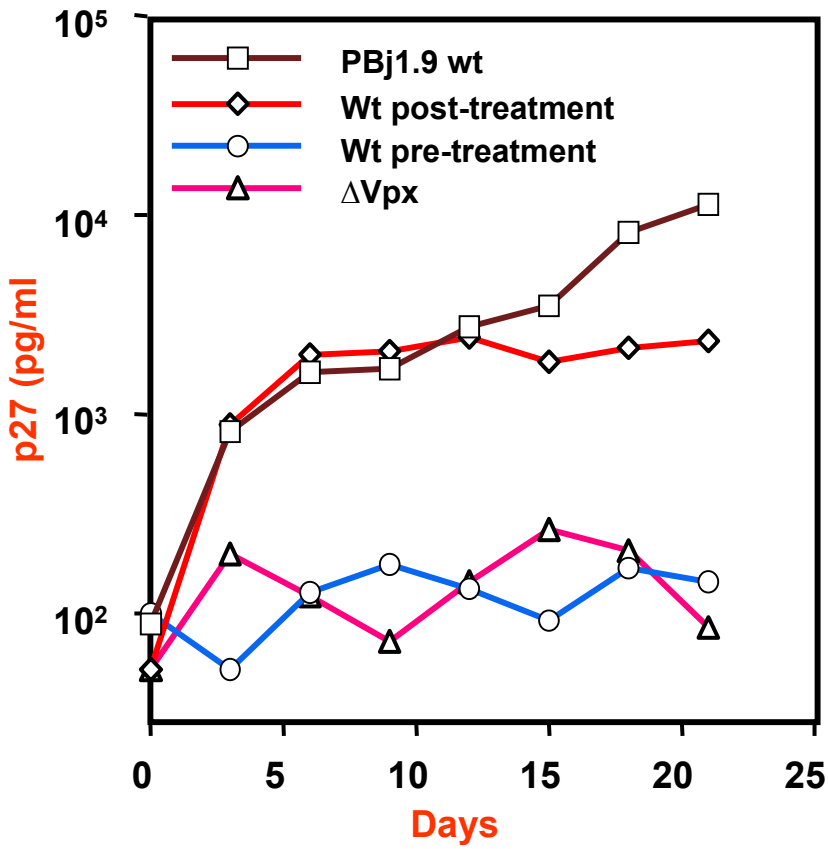

Figure 5.9: MAPK/ERK-2 modulates SIV infectivity. Terminally differentiated macaque macrophages were infected with SIVsmPBj1.9 virus (10 ng equivalent of $p27^{Gag)}$ produced in the presence or absence of MAPK inhibitor, hypericin. Virus replication was assessed by quantifying the amounts of $p27^{Gag}$ antigen in culture supernatants at 3-day post-infection intervals. Addition of hypericin to producer cells resulted in significant reduction in replication (Wt pre-treatment). Addition of hypericin to target cells resulted in inhibition of virus replication after 12 days (Wt post-treatment). wt, wild type: ΔVpx, Vpx mutant proviral clone; wt post-treatment, presence of MAPK inhibitor during and through out virus infection period; wt pre-treatment, viruses were produced in the presence of MAPK inhibitor.

Table 5.1: Effect of mutagenesis on phosphorylation and sub-cellular localization

Name	Phosphorylation status	Subcellular localization
Vpx wt	++	Nuc
Vpx S2L	++	Nuc
Vpx S13N	++	Nuc+Cyt
Vpx S52F	++	Nuc+Cyt
Vpx S63AS65A	+	Cyt
Vpx S2,13,52,63,65A	+/-	Cyt
Vpx T17I	++	Nuc+Cyt
Vpx T28I	++	Nuc+Cyt
Vpx T67A	++	Nuc+Cyt
Vpx T17,28,67A	++	Cyt
VpxS,T-A	+/-	Cyt
Vpx Y66A	++	Nuc
Vpx Y69A	++	Nuc
Vpx Y71A	++	Nuc
Vpx Y66,69A	++	Nuc
Vpx Y66,71A	++	Nuc
Vpx Y69,71A	++	Nuc
Vpx Y66,69,71A	++	Nuc
Vpx Y66,69,71P	++	Nuc
Vpx Y66,69,71S	++	Nuc
Vpx W24S	++	Nuc
Vpx W49S	++	Nuc
Vpx W53S	++	Nuc
Vpx W56S	++	Nuc
Vpx W49,53,56S	++	Nuc
Vpx W99S	++	Cyt
Vpx E30P	++	Nuc
Vpx H39L	++	Cyt
Vpx K68A	++	Nuc
Vpx L74,I 75S	+	Cyt
Vpx H82S	+	Cyt
Vpx G86C87S	++	Nuc+cyt
Vpx R100A	++	Cyt
Vpx P103, 106S	+	Cyt

++ Vpx detected; + low level of phosphorylation; +/- faint signal detected

presence or absence of hypericin and infectivity was examined in monocyte derived macaque macrophages. Virus particles produced in the presence of hypericin have shown significant reduction in infectivity almost similar to that of Δ *vpx* provirus (Fig. 5.9). Addition of hypericin had no apparent effect on virus maturation, production or release as indicated by similar levels of structural Gag proteins incorporated into the virus particles in the presence or absence of MAPK/ERK-2 inhibitor, hypericin (Fig. 5.8). Interestingly, addition of hypericin to target cells resulted in inhibition of viral replication 12 days after the infection (Fig. 5.9) suggesting that MAPK/ERK-2 regulates an early step in the virus life cycle, which is critical for establishment of viral infection in primary macrophages. Interestingly, we observed similar level of MAPK/ERK-2 incorporation into the virus particles in the presence or absence of hypericin (Fig. 5.8) indicating that hypericin blocks only MAPK/ERK-2 mediated phosphorylation of Vpx and subsequent virus replication but not virion incorporation of MAPK/ERK-2.

Chapter Six

Mechanism of Vpx incorporation into virus particles

Vpx is a virion-associated protein, packaged into the virus particles in equimolar amounts to that of structural polyprotein $Pr55^{Gag}$. In the virus particles Vpx seems to be localized within virus core and is incorporated efficiently in the progeny virions formed in the absence of *pol* and *env* gene products and is independent of viral RNA encapsidation. This indicates that expression of Gag precursor (Pr55Gag) is sufficient to mediate the incorporation of Vpx into virions. Studies have shown that Vpx is packaged into the virus particles by interacting with C-terminal p6 domain of Gag polyprotein. It was also shown that a leucine triplet (LXX)3 in p6 region is critical but not essential for Vpx incorporation as deletion of all the leucine repeats affects but does not abolish the incorporation of Vpx suggesting the involvement of other regions in Gag polyprotein. The exact mechanism involved in Vpx virion incorporation and also the amino acids that are required for virion incorporation of Vpx was not known. This part of the study was designed to understand the exact mechanism of Vpx virion incorporation by determining the following (a) the intracellular sites for Vpx/Gag interaction, (b) amino acids residues involved in Vpx/Gag interaction, and (c) the involvement of upstream regions in Gag polyprotein apart from C-terminal p6 domain.

6.1. Vpx is a virion associated protein

In order to understand the mechanisms (s) involved in virion incorporation of Vpx, we first checked for the presence of Vpx in purified virus particles. SIVsmPBj1.9 wild type and SIVsmPBj1.9 Δ *vpx* (Δ Vpx, a mutant in which the initiating and the internal methionine codons were replaced with threonine and leucine residues and contained a stop codon at amino acid position 80) proviral DNAs were transfected into 293T cells as descried in Materials and Methods. The culture supernatants were collected 48h after transfection, centrifuged over a 20% sucrose cushion, and the viral pellets were subjected to SDS-12% PAGE followed by Western blot analysis. Probing with anti-Vpx

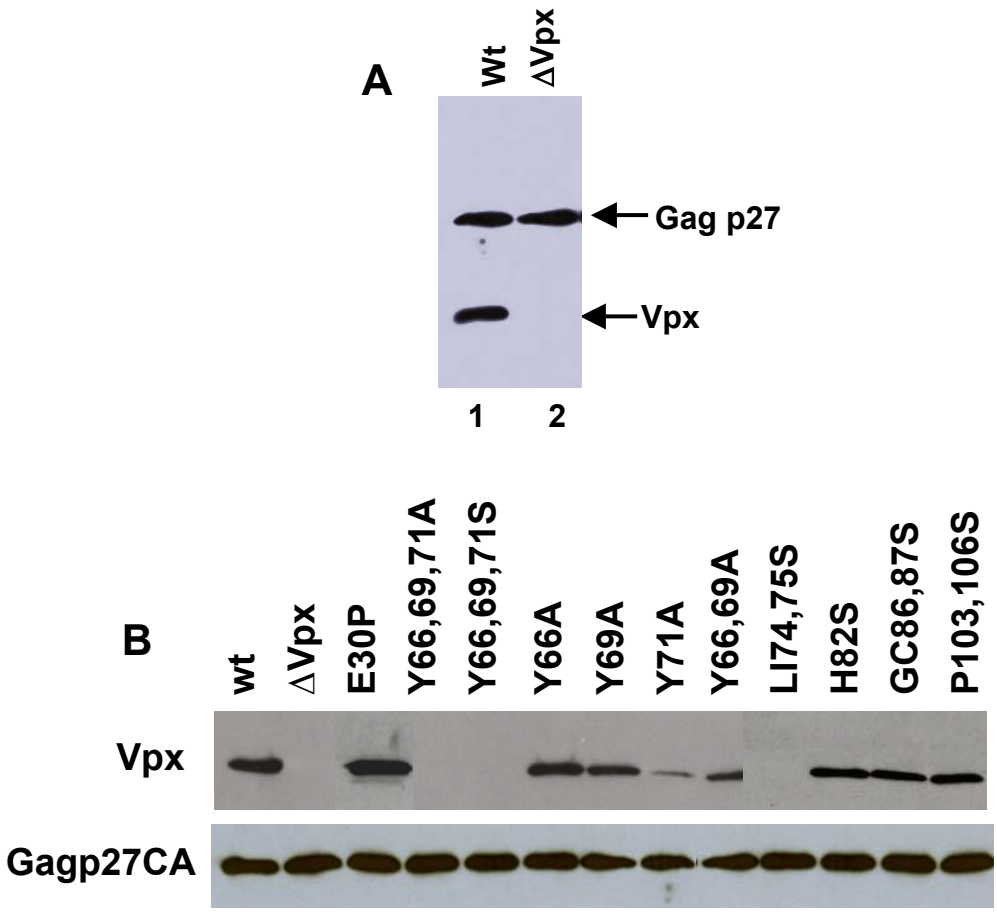

Figure 6.1: Vpx is packaged into SIVsmPBj1.9 virions. A. 293 T cells were transfected with wild type and Δ Vpx proviral clones. Viral particles were concentrated from culture supernatants by ultracentrifugation through a 20% sucrose cushion. The viral pellets were solubilized in gel loading buffer and resolved on a SDS-12% PAGE followed by Western blot using anti-Vpx and anti-Gag monoclonal antibodies. (Δ Vpx is a control construct lacking functional *vpx* open reading frame). **B.** Mutations in helical domain affect Vpx virion incorporation. 293 T cells were transfected with wild type and mutant Vpx proviral clones. The culture supernatants were collected 48h after transfection and centrifuged over a 20% sucrose cushion to purify the virus particles. The viral pellets (normalized by p27 Gag content) were resolved on a SDS-12% PAGE followed by Western blot using anti-Vpx and anti-Gag monoclonal antibodies.

and anti-Gag monoclonal antibodies revealed the presence of Vpx-specific signal in the wild type SIVsmPBj1.9 (Fig. 6.1A, lane 1) whereas the absence of Vpx from SIVsmPBj1.9 Δ *vpx* transfected samples (Fig. 6.1A, lane 2) suggests that Vpx is selectively packaged into virus particles.

6.2. Mutations in the helical domains of Vpx affect its virion incorporation

In order to identify the determinants that are required for Vpx virion incorporation, we introduced point mutations into the *vpx* gene of the infectious molecular clone SIVsmPBj1.9. All the conserved amino acid residues like serine, threonine and tyrosine, the targets for phosphorylation were selected for mutagenesis. SIVsm (PBj 1.9) proviruses containing the variants of *vpx* were transfected into 293-T cells, and the virion incorporation ability of Vpx mutant proteins was assessed. Forty-eight hours after transfection, the cell culture supernatants were collected and clarified to remove cell debris. The supernatants were layered on a 20% sucrose cushion and the virus particles were purified by ultracentrifugation. The viral pellets (normalized by p27 Gag content) were examined by Western blot analysis. Probing with anti-Vpx antibody revealed the absence of Vpx signal from mutants Y66,69,71A and LI74,75S (Fig. 6.1B). Furthermore, probing with anti-Gag antibody indicated that mutations in Vpx did not alter the expression, maturation and virus particle release (Fig. 6.1B, p27 capsid)

Since Gag is known to form virus-like particles and is sufficient for packaging of Vpx, we have employed an assay where Gag and different mutants of Vpx were co-transfected into Cos-7 cells. Twelve hours after transfection the cells were labeled with ^{35}S methionine and cysteine for 6h as described in Materials and Methods. The cells were harvested in ice cold PBS and the supernatants were collected separately. The supernatants were clarified to remove cell debris and then concentrated through a centricon 30. Vpx and Gag were immunoprecipitated from the cell lysates and supernatant

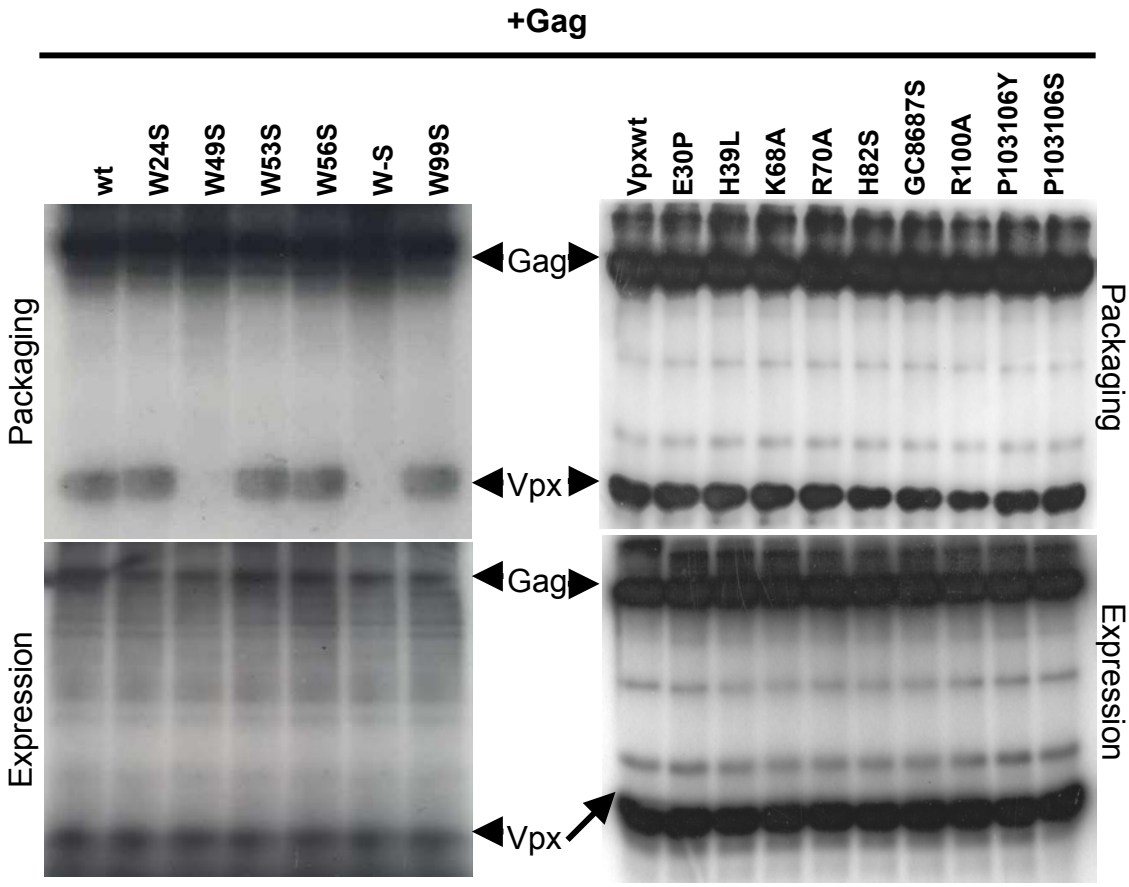

Figure 6.2: Mutations in helical domain II affect Vpx packaging into virus-like particles. Cos-7 cells were co-transfected with Gag and various Vpx mutant expression plasmids and 12h after transfection the cells were labeled for 6h with ^{35}S methionine and cysteine. The cells were harvested and supernatant was collected separately. Cells were lysed in lysis buffer and the supernatants were concentrated in a centricon 30. Gag and Vpx were immunoprecipitated from cell lysates and supernatant by using anti-Gag and anti-Vpx monoclonal antibodies respectively. After immunoprecipitation the samples were resolved on a SDS-15% PAGE followed by autoradiography.

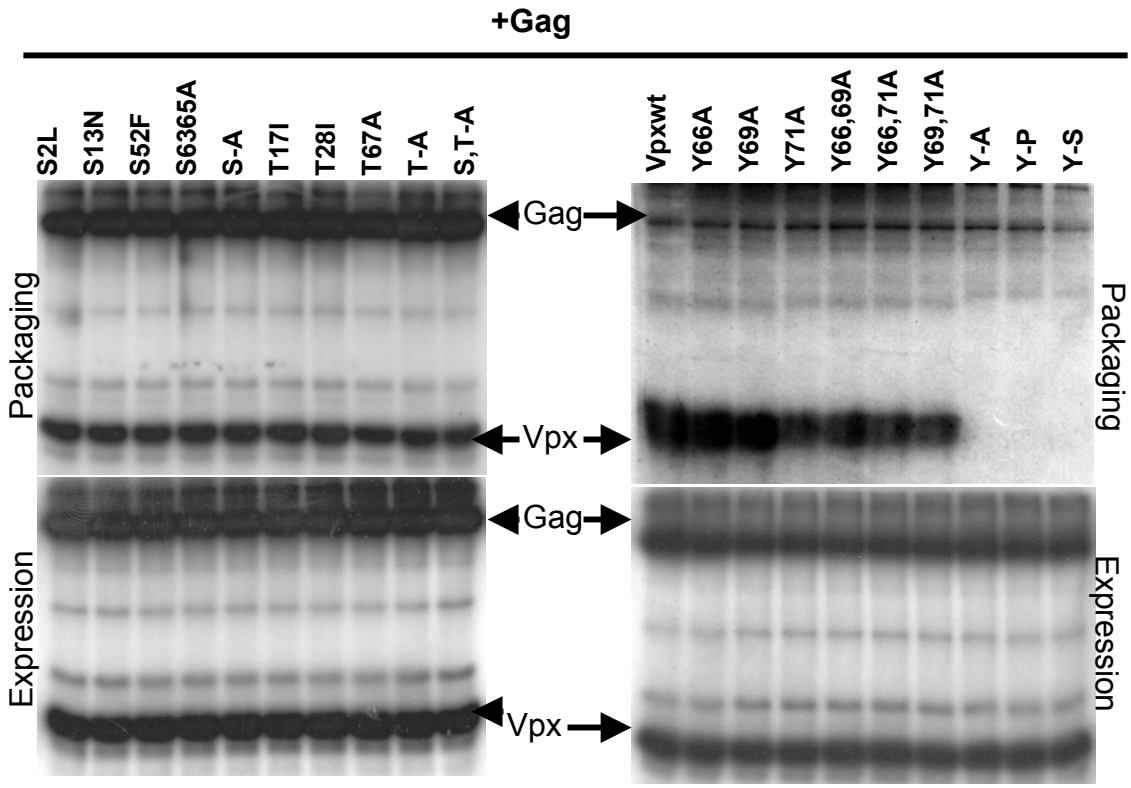

Figure 6.3: Substitutions in conserved tyrosines in helical domain III blocks Vpx packaging into virus like particles. Cos-7 cells were co-transfected with Gag and various Vpx mutant expression plasmids and 12h after transfection the cells were labeled for 6h with ^{35}S methionine and cysteine. The cells were harvested and supernatant was collected separately. Cells were lysed in lysis buffer and the supernatants were concentrated in centricon 30. Gag and Vpx were immunoprecipitated from cell lysate and supernatant by using anti-Gag and anti-Vpx monoclonal antibodies respectively. After immunoprecipitation the samples were resolved on a SDS-15% PAGE followed by autoradiography.

by using respective monoclonal antibodies, and the samples were resolved on SDS-15% PAGE followed by autoradiography. The results from figures 6.2 and 6.3 indicate the absence of Vpx signal from W49, W49,53,56S, Y66,69,71A, Y66,69,71P and Y66,69,71S mutants (packaging panel) even though equal levels of expression was found in cell pellets (expression panel). Introduction of helical destabilizing proline residue in the helical domain I (E30P) did not abrogate Vpx incorporation into virions as well as virus-like particles (Fig. 6.1 and 6.2) suggesting that the N-terminal helix I may be dispensable for Vpx incorporation. This is also true for mutations in the C-terminal region (amino acids between 82-112) like H82S, GC86,87S, R100A and P103,106S were packaged efficiently like wild type both into virions and virus-like particles (Fig. 6.1 and 6.2). Interestingly, when we analyzed the individual tyrosine mutants such as Tyr-66 and Tyr-69, they retained wild type virion incorporation, whereas exchange of Tyr-71 alone or in combination with either Tyr-66 or Tyr-69 has severely reduced Vpx incorporation into virus particles (Fig. 6.1B and 6.3). Collectively, these results suggest that amino acid residues Trp-49, Tyr-71, Leu-74 and Ile-75 may be involved in interactions with Gag, thereby facilitating Vpx incorporation. Interestingly, all these amino acids are located within predicted alpha helix II and III, suggesting that integrity of helix regions is critical for Vpx virion incorporation.

6.3. Phosphorylation is not required for Vpx virion incorporation

We described in chapter 5 that cellular kinase MAPK/ERK-2 phosphorylates Vpx and regulates Vpx nuclear import and virus replication in non-dividing macrophages. In order to understand whether phosphorylation modulates Vpx virion incorporation, we tested the virion incorporation ability of non-phosphorylated Vpx proteins. Since Gag expression was shown to be to sufficient to mediate Vpx incorporation into virions, we co-transfected Vpx and Gag expression plasmids into Cos-7 cells. Transfected cells were treated with inhibitors of MAPK and tyrosine kinase pathway alone or in combination and

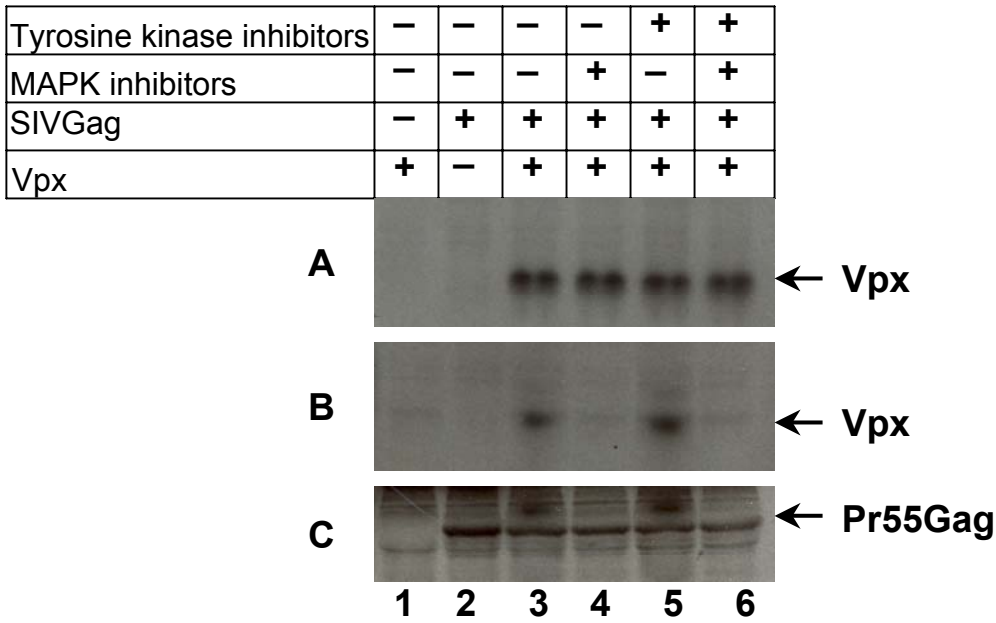

Figure 6.4: Phosphorylation of Vpx is not essential for incorporation into virus particles. Vpx and Gag expression plasmids were co-transfected into Cos-7 cells. Transfected cells were treated with inhibitors of MAPK pathways (hypericin and PD98059) and tyrosine kinases (PP2, genestein) alone or in combination and labeled with ^{32}P orthophosphoric acid or ^{35}S methionine and cysteine. Vpx efficiently incorporated into virus particles and is not altered by inhibitors of MAPK pathway or tyrosine kinases (panel A, lane 4 and 5). Whereas inhibitors of MAPK pathway blocked Vpx phosphorylation (panel B, lane 4) without altering its incorporation into virus-like particles (panel A, lane 4) indicating that phosphorylation is not required for Vpx incorporation into virus-like particles. Virus-like particle release (Pr55 Gag) is not affected by kinase inhibitors (panel C)

labeled with ^{32}P orthophosphoric acid and ^{35}S methionine and cysteine for 6h. The supernatant was collected and concentrated in a centricon 30. Gag and Vpx proteins were immunoprecipitated with respective monoclonal antibodies and the samples were resolved on a SDS-15% PAGE followed by autoradiography. Results in figure 6.4 indicate that Vpx was efficiently incorporated into virus-like particles generated by $Pr55^{Gag}$ precursor and was not altered by either inhibitors of MAPK pathways or tyrosine kinase alone or in combination (Fig. 6.4; panel A, lanes 4,5 and 6). Interestingly, inhibitors of MAPK pathway blocked Vpx phosphorylation (Fig. 6.4, panel B, lane 4) where as tyrosine kinase inhibitor did not inhibit phosphorylation (Fig. 6.4, panel B, lane 5). Furthermore, we observed equal amount of virus-like particle release (Fig. 6.4, panel C) in the presence or absence of kinase inhibitors suggesting that inhibition of Vpx phosphorylation did not alter virus-like particle release. Together, these results suggest that phosphorylation is not required for Vpx incorporation into virus particles.

6.4 Vpx interacts with P55 Gag in the cytoplasm

Gag was shown to be a nucleo-cytoplasmic shuttling protein and is sufficient to mediate Vpx incorporation into virions, but the underlying mechanisms of Vpx and Gag interaction inside the cells remain elusive. We therefore analyzed the *in vivo* interaction between Vpx and Pr55 Gag by using indirect immunofluorescence assay. Results in figure 6.5 indicate that Vpx co-localizes with Gag precursor in the cytoplasm, this suggest that these two proteins interact *in vivo.* This interaction was specific, as no co-localization observed between green fluorescent protein and Gag (Fig. 6.7). Further, to study this issue in detail we analyzed the relationship between co-localization of Vpx with Gag and Vpx virion incorporation. Various Vpx mutants were co-expressed in combination with Flag-$Pr55^{Gag}$ in Vero cells and their localization was visualized by indirect immunofluorescence assay using anti-Flag polyclonal antibody (for Gag) and anti-Vpx monoclonal antibody. Mutants like

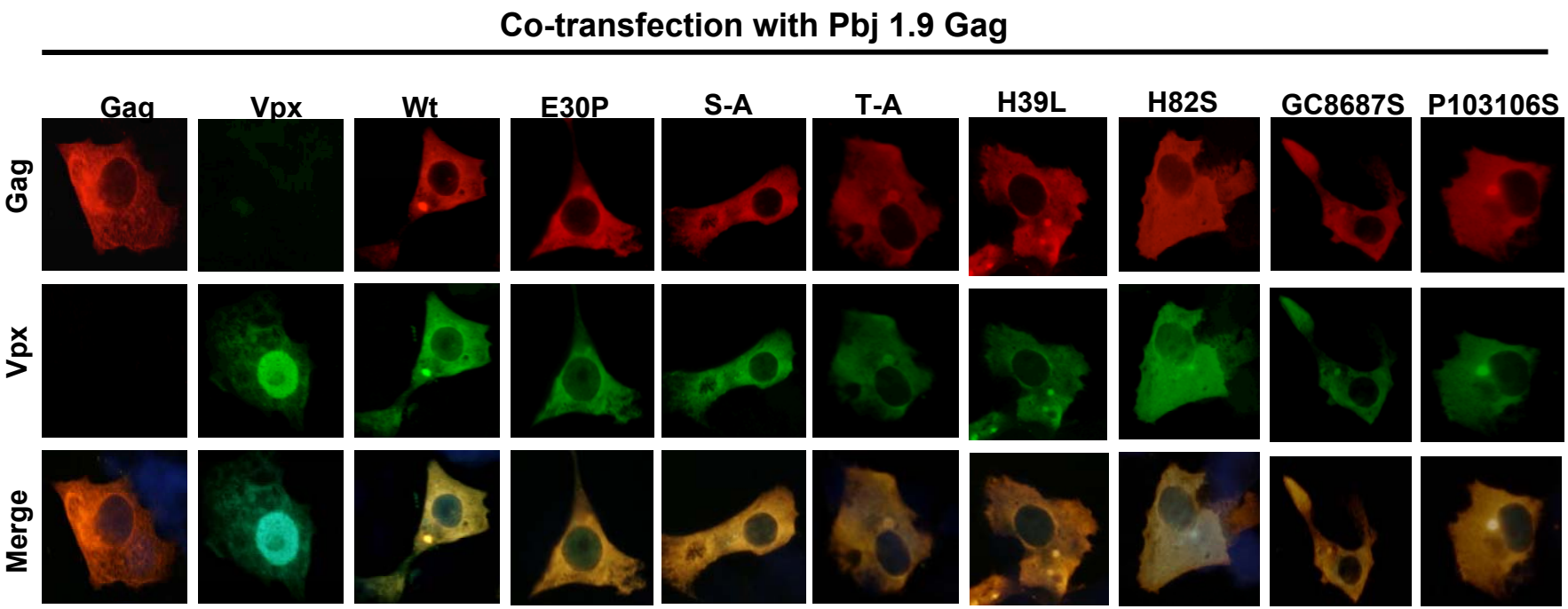

Figure 6.5: Vpx interacts with Pr55^{Gag} in the cytoplasm.vTF7-3 infected Vero cells were co-transfected with various Vpx mutant expression plasmids and Flag-Gag. Localization of Vpx and Gag proteins was detected by indirect immunofluorescence with an anti-Vpx monoclonal antibody and anti-Flag polyclonal antibody for Flag-Gag, followed by an goat anti-mouse Alexa fluor 488 conjugated secondary antibody (for Vpx) and goat anti-rabbit Alexa fluor 594 (for Flag-Gag). Green indicates localization of Vpx and red indicates localization of Gag. (Vpx S-A-serines at 2, 13, 52, 63, 65 changed to alanine and Vpx T-A threonines at 17, 28, 67 changed to alanine).

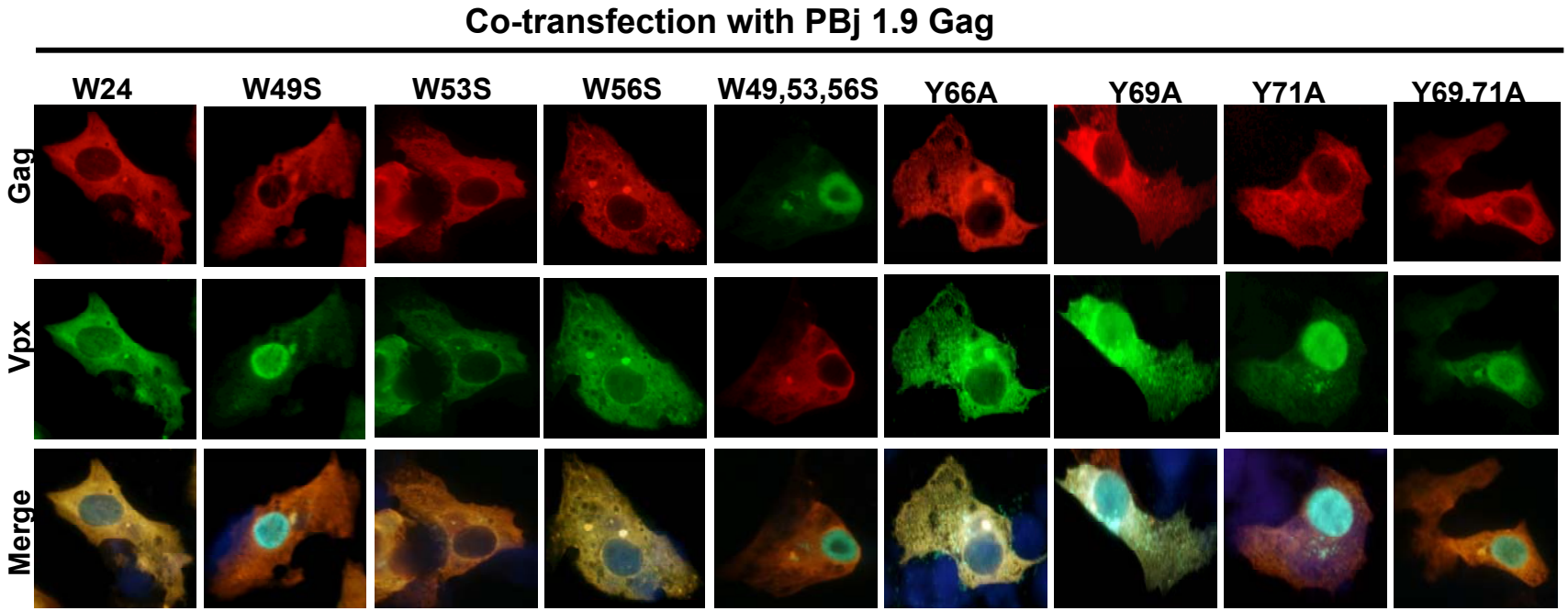

Figure 6.6: Conserved tyrosines and tryptophan residues are critical for Vpx co-localization with Gag in the cytoplasm.vTF7-3 infected Vero cells were co-transfected with various tyrosine and tryptophan Vpx mutant expression plasmids and Gag. Localization of Vpx and Gag proteins was detected by indirect immunofluorescence with an anti-Vpx monoclonal antibody and anti-Flag polyclonal antibody for Flag-Gag, followed by an goat anti-mouse Alexa fluor 488 conjugated secondary antibody (for Vpx) and goat anti-rabbit Alexa fluor 594 (for Flag-Gag). Green indicates localization of Vpx and red indicates localization of Gag.

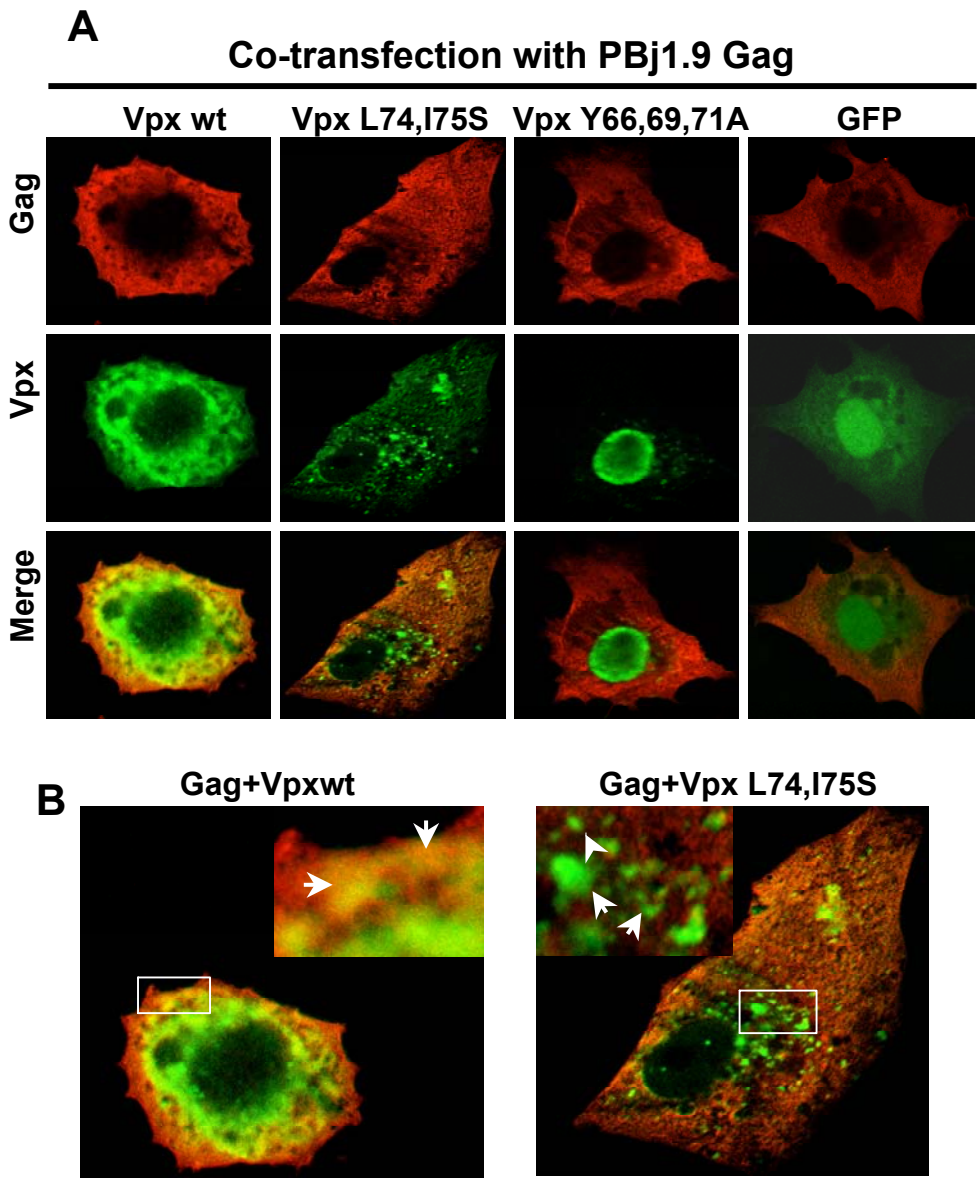

Figure 6.7: Vpx interacts and co-localizes with Gag precursor p55 in the cytoplasm. A. Variants of Vpx expression plasmids were co-transfected with the Gag expression plasmid in vTF7-3 infected Vero cells. Localization of Vpx and Gag proteins was detected by indirect immunofluorescence with an anti-Vpx monoclonal antibody and anti-Flag polyclonal antibody for Flag-Gag, followed by an goat anti-mouse Alexa fluor 488 conjugated secondary antibody (for Vpx) and goat anti-rabbit Alexa fluor 594 (for Flag-Gag). Green indicates localization of Vpx and red indicates localization of Gag. GFP is used as a negative control to show the specificity of interaction. **B.** Vpx specifically interacts and co-localizes with SIV Gag in the cytoplasm (indicated by yellow, inset box). Vpx L74,I75S did not co-localize with Gag despite its localization in the cytoplasm (indicated by separate red and green, inset box).

W49S, W49,53,56S, Y71A, Y66,71A, Y6971A and Y66,69,71A did not co-localize with Gag despite retaining wild type nuclear localization (Fig. 6.6) and were not incorporated into virus like particles (Fig. 6.2 and 6.3). Interestingly, the Vpx mutants (H39L, H82S, G86C87S and P103106S) that are defective for nuclear import but co-localize with Gag in the cytoplasm (Fig. 6.5) and retained the ability to incorporate into virus-like particles like wild type protein (Fig. 6.2). Taken together, these results suggest that Vpx interacts with Gag in the cytoplasm and is translocated to the site of virus assembly for virion incorporation. On the other hand, substitution of Leu-74 and Ile-75 resulted in cytoplasmic localization of mutant protein but failed to co-localize with Gag (Fig. 6.7A and 6.7B) and subsequently did not incorporate into virus particles (Fig. 6.1B) suggesting that these residues are critical for Vpx and Gag interaction.

6.5. Vpx interacts with p27 capsid and p6 portion of Gag

Vpx is localized with core p27 inside the virus particles and previous studies suggest that C-terminus of Gag *i.e.* p6 portion of p15 play a critical role in Vpx incorporation into virus particles. Since Vpx localizes within viral core, we determined whether Vpx interacts with viral capsid protein. In order to understand this mechanism, we expressed and purified the full length, matrix, capsid and p15 proteins of Gag as fusion with GST (Fig. 6.8A and 6.8B) and analyzed Vpx interactions by GST-pull down assay, followed by Western Blotting anti-Vpx monoclonal antibody. Results in figure 6.9A indicate that Vpx interacts with p55 and p15 as expected. Interestingly, we observed an interaction between Vpx and capsid similar to full length Gag whereas no interaction was noticed with Gag matrix. These results suggest that Vpx interact with capsid p27 in addition to p15 and this interaction may play a critical role in Vpx packaging into virus particles and subsequent viral infectivity in macrophages. Further, to understand the relevance of Vpx interaction with p27 capsid, we have selected various Vpx mutants with

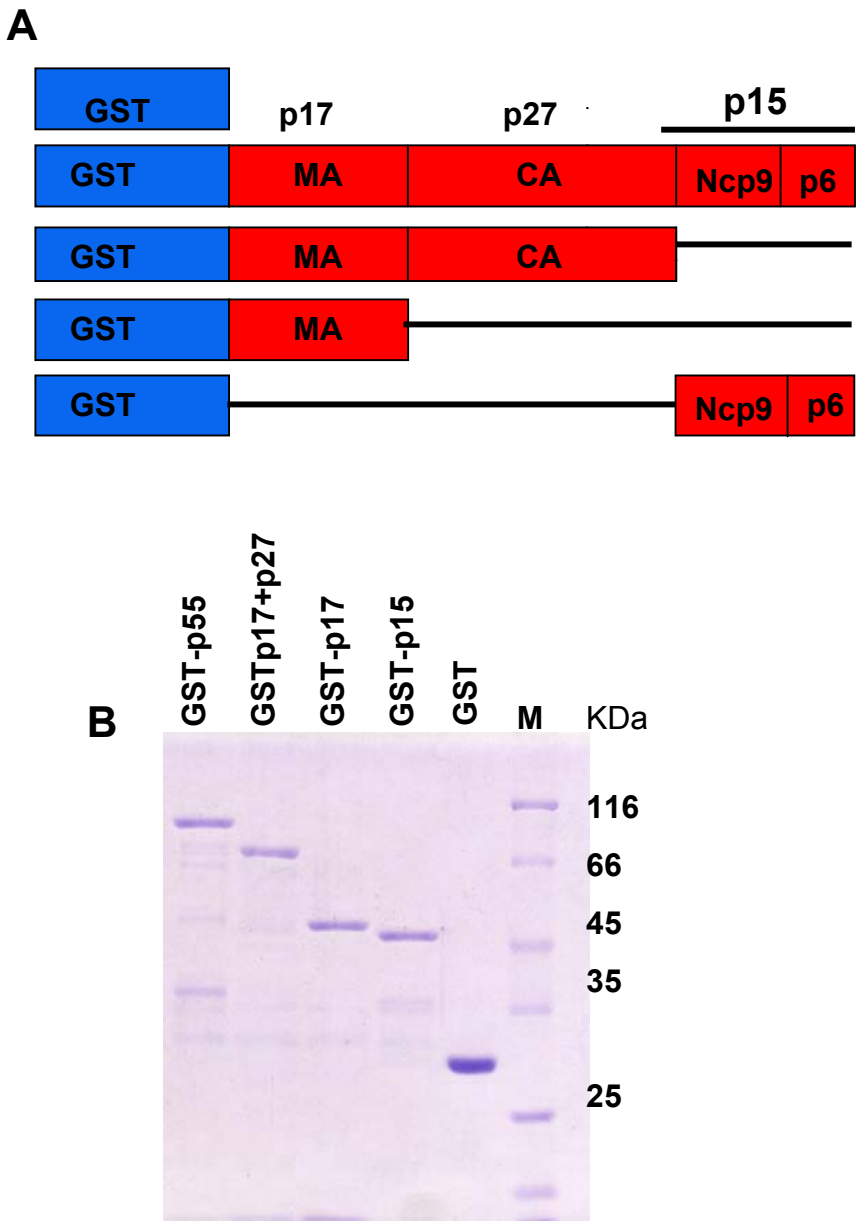

Figure 6.8: Construction and purification of full length, MA, CA and p15 of SIV Gag as GST fusion. A. Schematic representation of various GST-Gag constructs. **B.** Coomasie brilliant blue staining of purified GST, Gag p55, Gag-MA, CA, p15. GST fusion proteins were purified using GST-sepaharose beads as described in Materials and Methods.

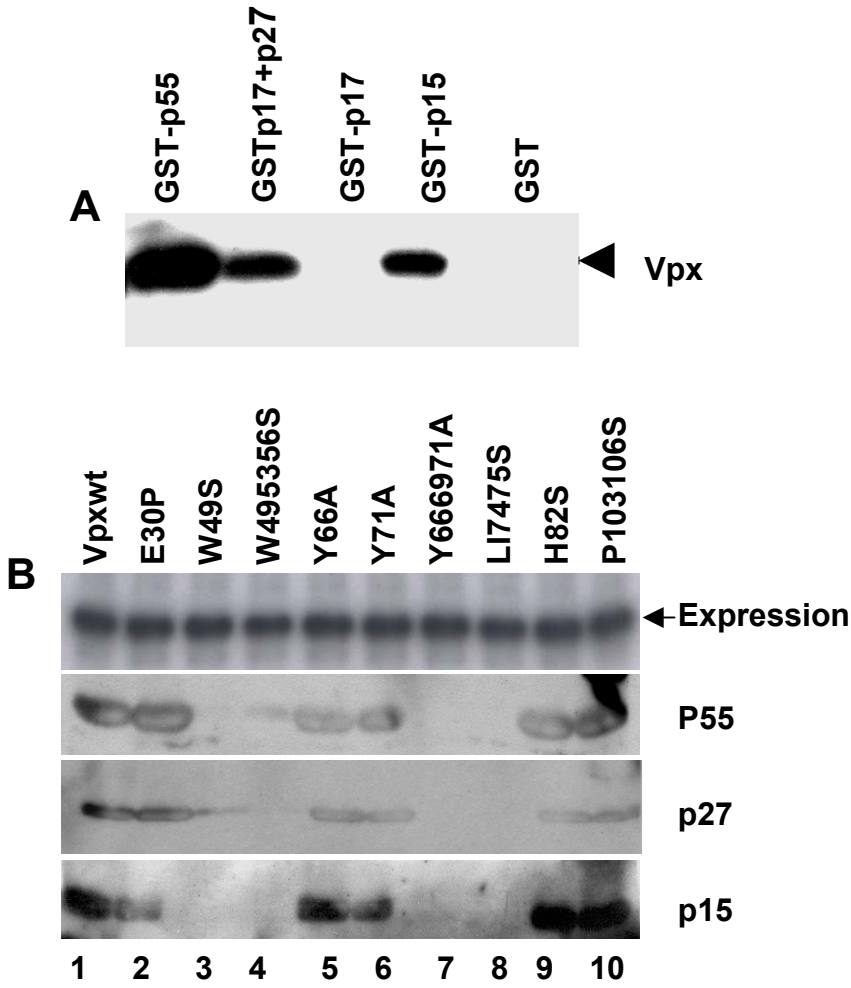

Figure 6.9: Vpx interacts with p27 CA and p15 portion of Gag. A. Cell lysate containing Vpx protein was mixed with sepharose beads bound with various GST-Gag proteins and incubated overnight. The bounded proteins were resolved on a SDS-12% PAGE followed by Western blot analysis using anti-Vpx monoclonal antibody. **B.** GST pull down assay using GST-Gag deletion constructs and various Vpx mutants followed by Western Blotting using anti-Vpx monoclonal antibody. Vpx mutants which did not package into virus-like particles or virus particles did not interact with p55, p27 and p15 *in vitro.*

different localization patterns and packaging abilities. We selected mutants with a) wild type localization pattern and incorporated into virus-like particles (E30P), b) mutants which are localized to the cytoplasm and packaged into the virus particles (H82S, P103,106S), c) mutants which localized to the nucleus and are not package into virus particles (W49S and Y66,69,71A) and d) mutant protein localized to the cytoplasm but not packaged into virus particles (L74,I75S). Interaction between Gag and Vpx mutants was analyzed by GST pull-down assay followed by Western blot using anti-Vpx monoclonal antibody. The results in figure 6.9 B indicate that mutants like E30P, Y66A, H82S, P103,106S that are packaged into the virus particles interact with both p27 and p15 (Fig. 6.9B lane 2, 5, 9 and 10). In contrast, mutants like W49S, W49,53,56S, Y71A, Y66,69,71A, L74I75S that are not packaged into virus particles did not show any interaction with either p27 or p15 (Fig. 6.9B lane 3, 4, 6 and 7). Together, these results suggest that Vpx interaction with p27 and p6 portion of p15 in $Pr55^{Gag}$ is critical for its efficient incorporation into virus particles.

6.6. Vpx is essential for efficient replication in macrophages

We next analyzed the ability of various *vpx* mutant proviruses to elicit a spreading infection in monocyte derived macaque macrophages. All *vpx* mutant viruses replicated efficiently and to high titers in CEMx174 cells (Fig. 6.10A). However, this was not the case in terminally differentiated macaque macrophage cultures. PBj1.9 mutants like Y66,69,71A and L74,I75S that failed to package Vpx proteins (Fig. 6.1B) were severely impaired in their ability to replicate in macrophages (Fig. 6.10B). Furthermore, failure to replicate in macrophages was also observed for mutants that were packaged into virus particles at near wild type quantities but showed an impairment in nuclear import (H82S, GC86,87S and P103,106S) (Fig. 6.1B and 6.10B). In three independent experiments PBj1.9 mutants with substitution in the carboxyl-terminal half of Vpx replicated poorly in macrophages, while the E30P

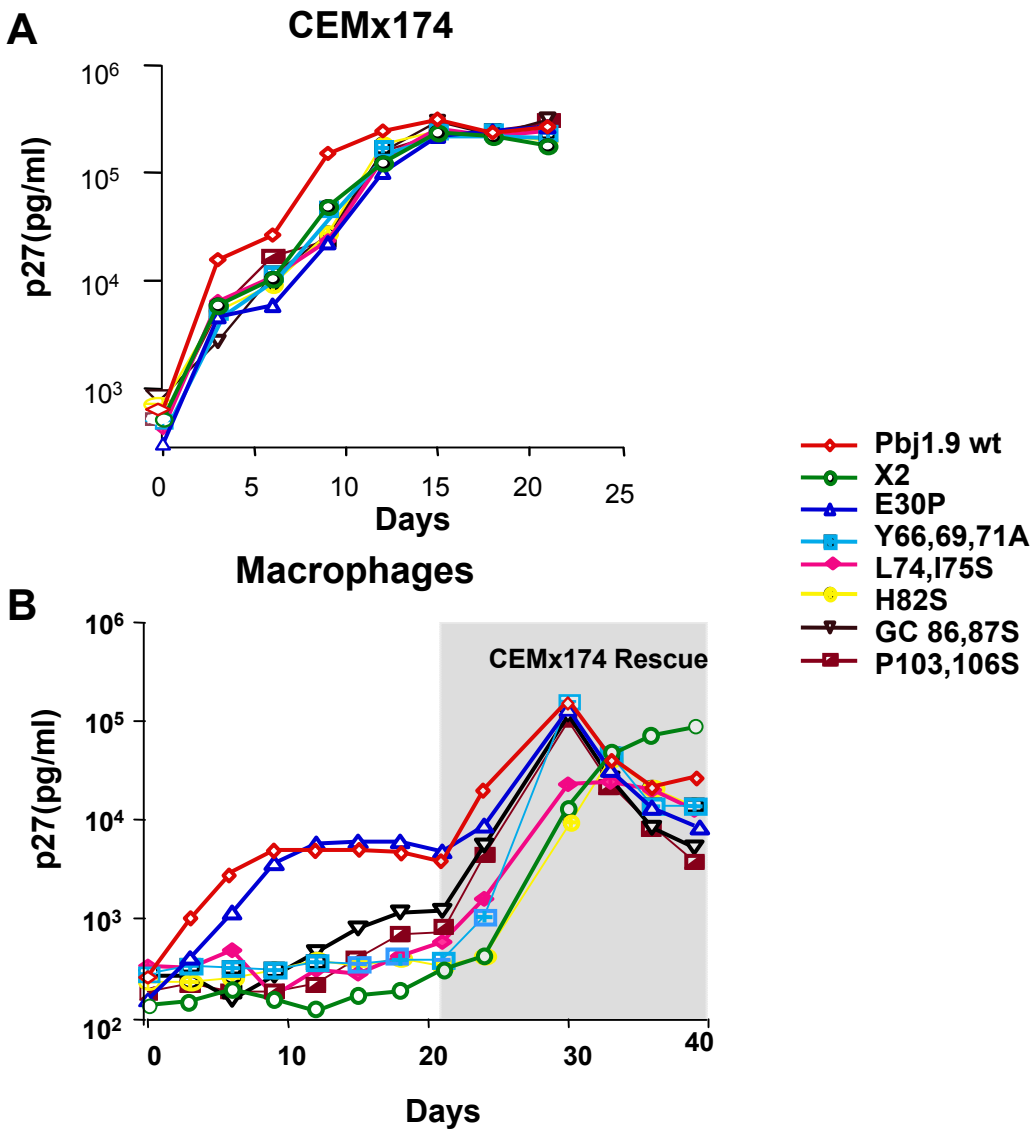

Figure 6.10: Vpx is critical for efficient infection in non-dividing macrophages. CEMx174 cells (**A**), and terminally differentiated macaque macrophages (**B**) are infected with indicated SIVsmPBj1.9 wild type and mutant proviruses equilibrated by p27 Gag (10ng of p27 Gag). Both wild type and mutant proviruses replicated well in dividing CEMx174 cells (**A**). In contrast Vpx mutant proviruses with defective Vpx nuclear transport (H82S, GC86,87S and P103,106S) and virion incorporation (Y66,69,71A, L74I75S) replicated poorly in non-dividing macrophages (**B**) Twenty one days after infection, macrophages were co-cultured with CEMx174 cells for 24 hours and the replication levels of Vpx mutant viruses were rescued indicating that Vpx is critical for virus replication in non-dividing macrophages.

mutation designed to disrupt the predicted N-terminal helix resulted in replication identical to that of wild type. Replication of all Vpx mutant viruses was rescued by CEMx174 co-cultivation with macrophages after 21 days (Fig. 6.10B). Collectively, these results suggest that presence of Vpx in the virus particles with ability to localize to the nucleus is critical for optimal virus replication in non-dividing target cells like macrophages.

Chapter Seven

Discussion

This is the first study to undertake the extensive mapping of the determinants that are required for Vpx nuclear localization and to define the interrelationship between phosphorylation, nuclear import and virion incorporation with relevance to viral replication. HIV-2/SIVsm Vpx has two distinct localization properties that direct it either to the nucleus (Pancio and Ratner, 1998; Mahalingam *et al*., 2001; Di marzio *et al*., 1995) or, in association with Gag, to budding virus particles at the plasma membrane (Henderson *et al*., 1988; Kappes *et al*., 1989; Accola *et al*., 1999; Wu *et al*., 1994). These distinct localization properties of Vpx may be mediated through different protein-protein interactions. In order to identify the determinants that are involved in nuclear import of Vpx, we introduced a number of point mutations into Vpx structural domains, which are highly conserved among divergent isolates of HIV-2 and SIV (Fig. 4.1D). Our results suggest that Vpx encompasses a novel determinant for nuclear localization and the nuclear import function of Vpx is critical for efficient virus replication in macrophage cultures. This was supported by a) exchange of serines and threonines impaired Vpx nuclear localization (Fig. 4.5B), b) a fragment of 20 amino acid residues (61-80) was found to be necessary and sufficient to mediate the import of Vpx as well as heterologous cytoplasmic proteins into the nucleus (Fig. 4.13) and c) mutations that are capable of affecting the ability of Vpx protein's nuclear import did not support the virus replication in primary macaque macrophages (Fig. 4.14). Virion incorporation occurs late in the infection when *de novo* synthesized Vpx is re-routed to the plasma membrane for packaging into budding virions through its interaction with Gag precursor polyproteins. Hence, the nuclear localization of Vpx is likely relevant early in the infection when Vpx found in the virion helps to translocate the viral genome into the nuclear compartment.

In this study, we have used confocal microscopic analysis to first check the localization of wild type Vpx *in situ*. Such analysis demonstrated that wild

type Vpx protein is efficiently translocated to the nuclear membrane and to the nuclear interior (Fig. 4.3) even in the absence of other viral encoded proteins. Given the nuclear accumulation of the wild-type protein, we sought to characterize cellular localization of different Vpx variants and to map the domain responsible for the nuclear import of Vpx. A striking finding of these experiments was that mutations in several widely separated regions affected Vpx nuclear localization. Exchange of serines and threonines has resulted in cytoplasmic localization whereas tyrosine and tryptophan mutants retain wild type localization (Fig. 4.5, 4.7 and 4.8). One of the critical regions was the conserved C-terminal domain between amino acids 61-80 in which mutations resulted in complete loss of the nuclear localization of Vpx (Fig. 4.6). Furthermore, C-terminal domain containing amino acids 61-80 of Vpx targeted β-Gal, a cytoplasmic protein into the nucleus indicating that Vpx has a novel nuclear transferable localization signal. The 20 amino acid minimal nuclear targeting signal identified overlaps with the helical domain III (amino acids 64-82) and is conserved among divergent isolates of HIV-2/SIVsm, such conservation of this motif and its sequence is highly indicative of the domain being important for Vpx function. Nuclear import of proteins in general, is a multi-step process where proteins are first targeted to nuclear pore complex (NPC) and subsequently translocated to the nuclear interior. This appears to be the case for Vpx, which is targeted to the nuclear membrane and subsequently imported into nuclear interior as observed by its localization patterns in this study. Similar results were also reported for HIV-1 Vpr (Jenknis *et al*., 1998; de Noronha *et al*., 2001). Though the minimal targeting signal is located between amino acids 61-80, the substitution of histidine 82 with serine has impaired nuclear import of Vpx suggesting that the integrity of putative helix III (amino acids 64-82) is important for efficient transport of Vpx into the nucleus. Helical domains are known to be involved in protein-protein interactions, protein-nucleic acid interactions (Saier and McCaldon, 1988; Subbramanian *et al*., 1998, Tacke *et al*., 1993). Interestingly, alteration of

helical domain I in HIV-1 Vpr blocked its nuclear import (Vodicka *et al*., 1998; Mahalingam *et al*., 1997; Sherman *et al*., 2001) suggest the critical role for helical domains in protein nuclear transport.

Recently, Pancio and co-workers reported that deletion of the C-terminal proline-rich domain (amino acids 102-112) in Vpx resulted in a block of nuclear localization of HIV-2 DNA, thus implicating that this domain is essential in the nuclear import of the HIV-2 PIC (Pancio and Ratner, 1998). The 20 amino acid nuclear targeting domain identified in this study lies upstream of the proline-rich region raising the possibility that like HIV-1 Vpr, Vpx may contain two independent nuclear targeting domains (Jenkins *et al*., 1998; Kamata and Aida 2000). Alternatively the deletion of proline-rich domain may have impaired Vpx function by changing structural conformation of the protein. To distinguish between these two possibilities, we fused the C-terminal proline-rich domain (PGPPPPPPPGLA) of Vpx to β-Gal and tested whether this proline-rich domain can mediate the nuclear import of a heterologous protein. Our results clearly indicate that C-terminal proline-rich domain is not absolutely required for nuclear import of Vpx (Fig. 4.11). This was further supported by confocal microscopic analysis of chimeric protein β-Gal/Vpx 81-112, containing the proline-rich domain of Vpx localized only in the cytoplasm whereas β-Gal/Vpx 1-80 localized to the nucleus (Fig. 4.12). These results suggest that proline-rich domain is not required for Vpx nuclear import and the minimal nuclear localization signal (NLS) identified in this study is necessary and sufficient to translocate Vpx to the nucleus.

We next assessed the role of Vpx nuclear import on replication potential of SIVsmPBj1.9 proviruses in dividing and non-dividing cells. Viruses that encoded the nuclear import defective Vpx protein failed to replicate or grew poorly in macaque macrophage cultures. For example, H82S and P103, 106S mutations impaired Vpx nuclear import and abrogated mutant protein's ability

to support virus replication in primary macaque macrophages. The reduced efficiency of Vpx mutant virus replication in macrophages even in the presence of other NLS containing viral proteins like Gag matrix and integrase supports the notion that nuclear import of Vpx is critical for the efficient replication of HIV-2/SIV in macrophages. This is in agreement with current consensus that suggests Vpx is the major nucleophilic determinant coded by HIV-2/SIV (Pancio *et al*., 2000; Fletcher *et al*., 1996).

Nuclear transport of NLS containing proteins is regulated by multiple mechanisms of which phosphorylation is the major regulatory mechanism. Phosphorylation plays a critical role in NLS mediated nuclear transport, cell cycle progression and gene expression (Fridell *et al*., 1997; Jans and Hubner, 1996; Peterson and Schreiber, 1999; Schakney and Shankey, 1999). Phosphorylation-regulated NLS were found to control nuclear transport in eukaryotic cells from yeast and plants to higher animals. Recent studies have demonstrated that serine-threonine kinases like MAPK/ERK-2 and cAMP dependent protein kinase A (PKA) of the host cell are incorporated within HIV-1 particles (Camaur *et al*., 1997; Gallay *et al*., 1995; Luo *et al*., 1997; Paul and Jabbar, 1997; Jacque *et al*., 1998) and regulate early steps in the viral life cycle. Having demonstrated that Vpx is the critical determinant for HIV-2/SIV replication in non-dividing cells, we studied whether nuclear transport of Vpx is regulated by phosphorylation. To this end, we first determined the presence of cellular MAPK/ERK-2 in the SIV particles and the phosphorylation status of Vpx. Our results suggest that Vpx is phosphorylated by virion associated MAPK/ERK-2 both *in vitro* and *in vivo* (Fig. 5.1 and 5.2). Recent reports suggested that hypericin efficiently inhibits MAPK activity (Jacque *et al*., 1998). We observed inhibition of Vpx phosphorylation when Jurkat cells were treated with hypericin suggesting that Vpx is a substrate for MAPK/ERK-2 (Fig. 5.3A). Furthermore, inhibition of MAPK/ ERK-2 activity by hypericin was specific as there was no effect on the activity of JNK (serine-threonine kinase), in the

presence of hypericin (Fig. 5.3B). Exchange of potential phosphorylation residues like serines and threonines in Vpx has resulted in cytoplasmic localization of mutant proteins suggesting that phosphorylation may regulate Vpx nuclear transport. To study this issue in detail, we have analyzed the presence of Vpx protein in different cellular compartments in the presence or absence of MAPK inhibitor, hypericin. Interestingly, we observed accumulation of Vpx in the nuclear fraction in the absence of hypericin whereas more Vpx protein was found in cytoplasm when transfected cells were treated with hypericin (Fig. 5.4A). This result was further supported by cytoplasmic localization of Vpx in the presence of inhibitors, hypericin or PD98059 (MEK inhibitor) (Fig.5.B). Collectively, these results suggest that MAPK/ERK2 mediated phosphorylation regulates Vpx nuclear transport. We also found that impairment of host cell MAPK/ERK-2 activity by hypericin resulted in the production of virions with reduced infectivity as assessed by infection assays performed in macaque macrophages (Fig. 5.9). Western blotting analysis of virion protein contents from cells exposed to MAPK inhibitor, hypericin revealed that it had no apparent effect on virus production or particle release from the infected cells (Fig. 5.8). These results suggest that the defects in viral infectivity may be due to the inhibition of virus-associated MAPK/ERK-2 activity, which may be critical for Vpx-mediated nuclear translocation of viral genome.

Our studies indicate that host cell MAPK/ERK-2 is selectively incorporated into virus particles and regulates SIV infectivity by modulating Vpx phosphorylation. It is likely that the MAPK inhibitor blocks SIV infection by interfering with the nuclear import of Vpx, thereby restricting subsequent virus replication in macrophages. MAPK/ERK-2 is a proline directed kinase (Davis, 1993) but there are no consensus MAPK/ERK-2 sites within Vpx. Despite this, immunoprecipitates of MAPK/ERK-2 from cell lysates as well as recombinant kinase were able to phosphorylate Vpx. MAPK/ERK-2 phosphorylation of a

protein, which lacks MAPK consensus recognition sites, has been reported recently (Corbalan-garcia *et al*., 1996) for both viral and cellular proteins and suggested that conformation of the substrate protein was sufficient to allow recognition by MAPK. For example, Gag matrix protein of HIV-1, which lacks MAPK consensus recognition motifs was phosphorylated by MAPK/ERK-2 and regulates the transport of viral DNA to the target cell nucleus (Jacque *et al*., 1998). We also assessed the role of Vpx phosphorylation and nuclear transport on the replication potential of *vpx* mutant SIVsmPBj1.9 in non-dividing cells. Viruses that encoded phosphorylation and nuclear import defective Vpx mutant protein failed to replicate or grew poorly in macaque macrophage cultures despite wild type packaging ability suggesting that phosphorylation of Vpx is required for efficient nuclear import of viral genome and subsequent replication in non-dividing cells like macrophages. Together our data demonstrate that presence of Vpx in the virus particles with ability to transport into the nucleus is critical for efficient virus replication in macrophages.

Vpx is incorporated into the virus particles by interacting with p6 portion of Gag (Kappes *et al*., 1993; Wu *et al*., 1994; Pancio and Ratner, 1998) and is present in molar amounts equivalent to that structural protein Gag. A leucine triplet motif in the N-terminal region of p6 was shown to be critical for virion incorporation of Vpx (Selig *et al*., 1998). Our results indicate that the integrity of helical domains II and III is critical for efficient virion incorporation, as supported by the mutations within these domains (W49S, W49,53,56S, Y71A and Y66,69,71A) has resulted in complete abrogation of Vpx packaging into virus particles as well as virus-like particles (Fig. 6.1, 6.2 and 6.3). To further understand the mechanism of Vpx and Gag interaction, we analyzed the *in vivo* interaction between Gag and Vpx by using indirect immunofluorescence assay. Vpx when transfected alone localized to the nucleus but in combination with Gag it is localized in the cytoplasm (Fig. 6.6), this provides evidence that

these two proteins interact *in vivo*. In order to understand the relation between Vpx incorporation into virus particles and its localization with Gag inside the living cells, we co-expressed various mutants of Vpx with Gag and studied their localization. Comparison of results from immunofluorescence and packaging indicate that Vpx interaction with Gag in the cytoplasm is critical for the incorporation of Vpx into virions. Vpx mutants H82S, GC8687S and P103,106S which are co-localized with Gag in the cytoplasm are incorporated into virions (Fig. 6.2 and 6.5) whereas mutants like W49S, W49,53,56S and Y66,69,71A which retained nuclear localization but did not co-localize with Gag in the cytoplasm are not incorporated into virions. These results suggest that nuclear transport property of Vpx is not required for its incorporation into virus particles. Interestingly, W49S and Y66,69,71A mutants failed to package but retained wild type nuclear localization, suggesting that Vpx may be exported to the cytoplasm and export property of Vpx may be required for its availability in the cytoplasm for subsequent incorporation into virus particles like HIV-1 Vpr (Sherman *et al*., 2001; Jenkins *et al*., 2001; Subbramanian *et al*., 1998. Collectively, our data suggest that Vpx interaction with structural protein Gag in the cytoplasm is critical for its incorporation into budding virus particles to support efficient virus replication.

Recent reports suggest that efficient uncoating of viral core is essential for efficient viral genome nuclear transport and replication in non-dividing target cells (Yamashita and Emerman, 2004; Forshey *et al*., 2002). Since Vpx is found inside the virion core (Kewalramani and Emerman 1996), we asked a question whether Vpx interacts with core capsid p27 in addition to its interaction with p15 to support the uncoating process. In order to understand the Vpx interaction with Gag, we expressed and purified the full length, matrix, capsid and p15 proteins of Gag as fusion with GST and analyzed Vpx interactions by GST-pull down assay, followed by Western blot using Vpx monoclonal antibody. Our results suggest that Vpx interacts with p27 capsid

in addition to p15 portion of $Pr55^{Gag}$. In contrast, no interaction was observed with Gag matrix. These results are in accordance with previous studies where it was shown that SIVmac Gag lacking matrix p17 can still interact with Vpx in a yeast two hybrid assay (Selig et al., 1998). These data suggest that Vpx interact with core capsid p27 in addition to p15 and this interaction may play a important role in Vpx packaging into virus particles. It is reasonable to hypothesize that the observed interaction of Vpx with p27 CA may be critical for efficient uncoating of viral core in non-dividing cells like macrophages which may be required for optimal replication of HIV-2/SIV in macrophages.

Chapter Eight

Summary

The results presented in this thesis suggested the critical role of virus protein X (Vpx) in HIV-2/SIV replication as supported by the following evidence.

1. Vpx is 18 kDa protein transported into the nucleus independent of other viral proteins. Amino acids 61-80 of Vpx constitute a novel non-canonical transferable nuclear localization signal and capable of transporting a heterologous cytoplasmic protein into the nucleus. Exchange of the conserved residues within the minimal nuclear targeting domain abrogates Vpx nuclear import.

2. Vpx is phosphorylated by virion associated cellular kinase MAPK/ERK-2 both *in vivo* and *in vitro.* This phosphorylation is specifically inhibited by MAPK inhibitor, hypericin. Phosphorylation by MAPK/ERK-2 regulates Vpx nuclear transport as evidenced by cytoplasmic accumulation of Vpx in the presence of MAPK inhibitor, hypericin. Phosphorylation of Vpx by MAPK/ERK-2 modulates SIVsmPBj1.9 infectivity in non-dividing target cells like macrophages.

3. Vpx is a virion-associated protein and is packaged into the virus particles through its interaction with structural protein Gag. Integrity of helical domain II and III is essential for Vpx incorporation into and virus particles. Vpx interacts with Gag in the cytoplasm and is translocated to the sites of viral assembly i.e. plasma membrane. Vpx interaction with both p27 capsid and p6 portion of p15 is essential for efficient incorporation into the virus particles.

4. Vpx is the major determinant for efficient HIV-2/SIV replication in non-dividing target cells like macrophages. This is supported by a) impairment of Vpx nuclear import leads to reduction in replication in non-dividing cells, even in the presence of other known karyophiles like matrix and Vpr and b)

blocking of MAPK/ERK-2 mediated phosphorylation of Vpx by MAPK inhibitor, hypericin leads to cytoplasmic accumulation of Vpx and subsequent impairment of virus replication in macrophages.

5. Results in this study clearly suggest that presence of Vpx in the virus particles with wild type nuclear transport ability is critical for efficient HIV-2/SIV replication in macrophages.

The results in the present study support the current consensus that Vpx is the major determinant in the nuclear transport of HIV-2/SIV genome into the primary target cells like macrophages. Our results also provide new insights in understanding the mechanisms that govern the early steps in primate lentiviral infection, which may help in designing more efficient anti-viral molecules in combating HIV infection.

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