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Exploring the Early Events in the HIV-1 Life Cycle: From Post-Entry Restriction to Nuclear Import

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Exploring the early events in the HIV-1 life cycle: From post-entry restriction to nuclear import

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Abstract

Early events in the viral replication cycle of the human immunodeficiency virus (HIV) determine the virus' ability to sustain a persistent infection in susceptible host species. However, many of the molecular machinations through which the virus progresses during its earliest moments in the cell's interior remain enigmatic. Similar to other virus families, HIV virions are packed with a specific assortment of viral and cellular proteins that allow them to carry out their unique program for replication inside of target cells. One such protein that is present in incoming virions, HIV-1 integrase, is an enzyme that facilitates the fusion of the viral genome and the host genomic DNA in the nucleus, thereby making HIV a permanent fixture in infected individuals. We have discovered that, in addition to its role in catalyzing integration, the HIV-1 integrase plays a key part in transporting the viral DNA through the cytoplasm to the nucleus during a process known as viral nuclear import. Viral nuclear import is a key early step in the viral life cycle directly preceding integration that has to date not been exploited as a target for antiretroviral therapy. Our recent studies indicate that a small stretch of sequence within the carboxyl terminus of the HIV-1 integrase protein contains a nuclear localization signal (NLS) that is indispensable for viral nuclear import and therefore absolutely required for productive HIV infection. Importantly, we have identified several mutations

(NLS mutants) within this region that can disrupt nuclear import and therefore completely stall HIV infection. In addition, alignment of all the known subtypes of HIV within the region specified by our NLS mutants indicates that the virus seldom changes its sequence in this part of its genome, suggesting that this domain is a critical factor that it must preserve for optimal infection. Thus, a major focus of this thesis is to clarify the role that the HIV integrase plays in viral nuclear import. In turn, these analyses may provide a rational basis for the design of new experimental drug screening systems dedicated to finding inhibitors that block HIV-1 nuclear import and curtail HIV infection. A secondary aim of this work is to understand the mechanism of species-specific restriction of lentivirus infection in primates mediated by endogenous host cell restriction factors such as Lv1/TRIM5-α. Lastly, a potential role for non-pathogenic viral DNA episomal circles formed from integration-defective HIV virions as a novel platform for gene therapy based vaccines is discussed.

In memory of my great-grandmother, Mable Louise DeGraff

Acknowledgements

This thesis is dedicated to my grandmother, Alfreda DeGraff-Simmons, who is the main reason I chose to pursue a career in science in the first place. It was she who first introduced me to science and nurtured my passion for it as a child by frequently taking me to the laboratory in which she worked as scientist for the Federal Government for 33 years until she retired at the National Institutes of Health's National Cancer Institute in Bethesda, Maryland. I also dedicate it to my loving mother, Phyllis Cunningham, who in addition to bringing me into this world did an excellent job in guiding my educational career and academic development throughout my entire life. My biological father was not a part of my life, which placed an extra burden on my mother and grandmother to provide a loving home environment as well as give me access to the best educational opportunities possible. I cannot thank them enough for shouldering that burden and helping me grow and develop into the man that I am today. I also want to acknowledge the life and memory of my great-grandmother, Mable DeGraff, who instilled the values into my family that allowed us to prosper and achieve beyond our economic and social status as African-Americans in post-apartheid America.

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CHAPTER 1. INTRODUCTION

Background: Biology of HIV-1 Infection

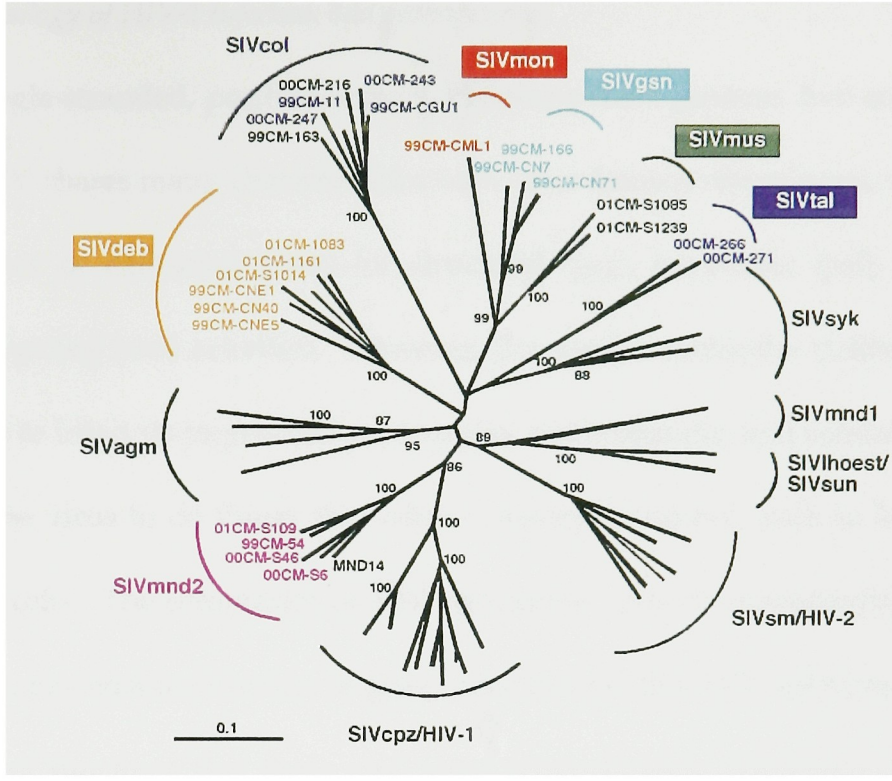
I. HIV-1 natural history, phylogeny, and structural organization

The human immunodeficiency virus (HIV) belongs to a family of viruses called the retroviridae, more specifically, to a subgroup known as lentiviruses. In 1983, HIV was discovered to be the etiological agent of acquired immunodeficiency syndrome (AIDS), a debilitating clinical condition which gradually erodes the immune system leading to increased susceptibility to opportunistic infections, and eventually, to death in infected patients. When HIV was first isolated by Luc Montagnier, Françiose Barre-Sinousi, William Rozenbaum and colleagues at the Pasteur Institute from a lymph node biopsy taken from a patient presenting the early clinical symptoms of AIDS, and shown by Robert Gallo and colleagues at the NCI to have reverse transcriptase activity (a hallmark of all retroviruses), it became only the second retrovirus discovered to infect humans (the first being the Human T-cell Leukemia Virus (HTLV I and II), whose discovery preceded that of HIV by just three years) [1, 2]. At first, HIV was thought to represent a new subtype of Human T-cell Leukemia virus, and was dubbed HTLV-III. However, while these two human retroviruses both share a characteristic tropism

of CD4-bearing cells, they differ greatly in physical appearance, molecular structure, and pathogenicity. It was later discovered that HIV more closely resembles other known animal lentiviruses (including equine infectious anemia virus EIAV, Visna virus, feline immunodeficiency virus FIV, and simian immunodeficiency virus SIV) in structure, cell tropism, and replication kinetics within infected cells [reviewed in [3, 4]]. These viruses, which derive their name from the Latin term for slow ("lenti-"), accordingly cause chronic, persistent infections that cause immune dysfunctions and neurological disorders in a diverse array of animals including sheep, horses, cats and monkeys. Consequently, the current global HIV epidemic is thought to have begun with a zoonotic transmission of an SIV virus from a primate species in Central Africa (most likely SIV*cpz* from chimpanzees) in an area where there is close contact between humans and non-human primates. Indeed, there is a high prevalence of these SIV viruses in feral populations of many African monkey species, including those strains related to SIV*cpz*, which is believed to have entered humans via the monkey butcheries found in locations where these animals are used as a food source for the native human population [5]. Figure 1 shows the phylogenetic relationships between the known SIV viruses and highlights the relatedness of SIV*cpz* to the current HIV-1 virus responsible for the current AIDS pandemic. Four different primate species are shown, rhesus macaques, chimpanzees,

humans, and owl monkeys, all of which have different susceptibilities to HIV infection depending upon the endogenous retroviruses (which provide the basis for the differentiation of host cell restriction factors like Lv1/TRIM5-α) that they naturally harbor. Incompatibility of cellular receptors for the virus envelope as well as varying levels of innate immunity to immunodeficiency viruses may also explain the differences in susceptibility to lentiviral infection among these primate species.

A.



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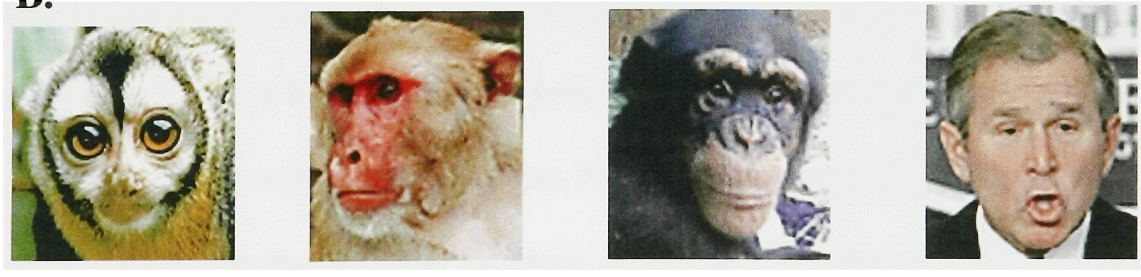


Owl Monkey (Aotus trivirgatus)

Rhesus Macaque (Macaca mulatta)

Chimpanzee (Pan troglodytes)

Human (Homo sapiens)

Figure 1. Natural history of HIV/SIV infection of primates. A) A 650-bp *pol* fragment was amplified from monkeys representing seven different primate species, sequenced, then subjected to phylogenetic tree analysis by the neighbor-joining method. The positions of 21 SIV sequences (in color) are shown in relation to HIV/SIV reference sequences from the Los Alamos HIV/SIV Sequence Database (in black). It is thought that the current HIV pandemic emanated from zoonotic transfer of such primate SIVs from practices such as bushmeat butchery. **B)** Pictures of an owl monkey (*Aotus trivirgatus*), rhesus macaque (*Macaca mulatta*), chimpanzee (*Pan troglodytes*), and human (*Homo sapiens*) are shown. Depending on the ancestral retroviruses that each of these primate species harbor, they have varying degrees of natural resistance to HIV or SIV viruses due to the presence or absence of antiviral factors such as Lv1/TRIM5-alpha. [Adapted from reference 240].

II. Molecular biology of HIV-1 infection and pathogenesis

With a single-stranded, positive-sense 9.8 kilobase RNA genome, two copies per virion, HIV shares many characteristics with other known retroviruses, such as the presence of dedicated genes for structural (*gag*), enzymatic (*pol*), and membrane targeting (*env*) activities. However, the specific molecular pathways that HIV uses to infect its target cells are complex and enigmatic, and combined, they enable the virus to do things that other retroviruses cannot, such as infect non-dividing cells. The complexity of HIV replication cycle is undoubtedly the result of the unprecedented number of genes, nine in total, that HIV specifies and the plurality of functions that these viral gene products carry out within host cells. In addition to the standard three retroviral genes (*gag*, *pol*, and *env*), the HIV genome encodes at least six other accessory genes (*tat, rev, nef, vif, vpr*, and *vpu/vpx)*, the most of any known retrovirus. Figure 2 shows the genetic organization of the HIV-1 genome with the locations of the currently known viral proteins indicated. As we gain more knowledge about the biological functions of all of HIV viral gene products, specifically how they interact with each other and host cell factors to promulgate productive infections with this virus inside of its target CD4+ cells, we will be better able to design new antiviral therapies to block its replication and eradicate AIDS. HIV virions are typically 100 nanometers in diameter and are indistinguishable from other lentiviruses under an electron

microscope. Their cone-shaped capsids contain the diploid viral genomic RNA along with associated viral enzymes reverse transcriptase,

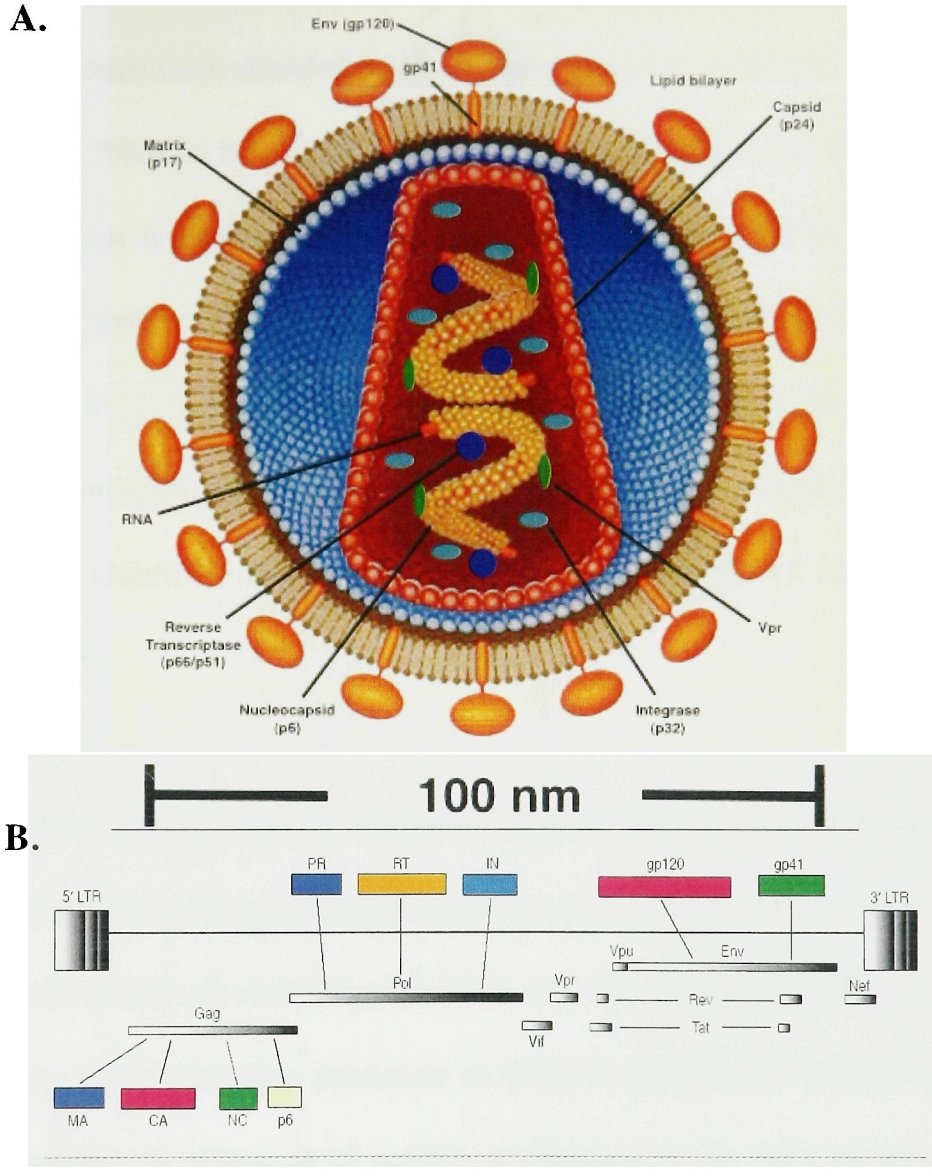


Figure 2. Virion structure and genetic organization of HIV-1. A) A cross-section of an HIV virion with the predicted location of all known viral proteins and phospholipid bilayer shown. **B)** The location of the long terminal repeats (LTRs) and the genes encoded by HIV-1 are indicated. Gag, Pol, and Env proteins are initially synthesized as polyprotein precursors. The Gag precursor is cleaved by the viral protease (PR) into the mature Gag proteins: matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC), and p6. The GagPol precursor undergoes proteolytic processing to generate the Gag structural proteins and the Pol enzymes: protease (PR, p10), reverse transcriptase (RT, p66/51) and integrase (IN, p32). The Env glycoprotein precursor, gp160, is cleaved by a cellular protease during transport to the cell surface to generate the mature surface glycoprotein gp120 and the transmembrane glycoprotein gp41. The sizes of the genes and encoded proteins are not drawn to scale. [Adapted from reference 239].

integrase, and protease. The viral capsid is surrounded by a phospholipids bilayer whose outside is studded with trimeric clusters of the viral envelope protein gp160. Prior to its migration to the plasma membrane, the full-length gp160 polyprotein is cleaved by cellular proteases into two distinct subunits, gp120 (surface antigen, SU) and gp41 (transmembrane, TM), and these two proteins form into non-covalently linked heterodimers which assemble into stable trimeric complexes once at the virion surface. A brief description of each of the viral genes identified to date and overview of their proposed function(s) is described below.

The HIV gag gene encodes a large 55 kD polypeptide which is cleaved by the viral protease into four smaller proteins: the p24 capsid protein (CA), the p17 matrix protein (MA), p9 nucleocapsid (NC), and the p6 protein. This 55 kD protein, often called the Gag precursor or Pr55, is myristylated at its amino terminal end, which allows it to remain embedded in the cells phopholipid bilayer at the interior cytoplasmic side while it is cleaved into p24, p17, p9, and p6 as the virions bud. Myristylation of the Gag precursor is required for the production of viable virions and mutants which are defective for Pr55 myristylation fail to replicate [6]. As the myristylated Gag precursor accumulates in increasing quantities at the plasma membrane, viral particles begin to form

and undergo the process of "budding" from the cell into many discrete virions [reviewed in [7]]. The full length viral genomic RNA is recruited to these membrane localized Gag precursor assemblies through its interactions with NC, which binds to the packaging sequence found in the 5′ end of the RNA [8]. The NC protein has also been shown to non-specifically bind RNA, and it is thought that the diploid RNA strands encapsidated in the virion are afforded a protective coating of NC protein.

As the viral particles bud from the plasma membrane, several viral proteins are responsible for creating the mature infectious virions that attack new uninfected cells. The most prominent of these is the HIV protease (PR) which auto-catalytically releases itself, then cleaves the Gag-Pol precursor protein into its active components MA, CA, NC, p6 and the viral enzymes reverse transcriptase (RT) and integrase (IN), and more PR [reviewed in [9, 10]]. The p17 MA protein aids in the formation of mature infectious virions by stabilizing the envelope protein at the plasma membrane through its binding of the cytoplasmic tail of gp41 [11]. Recent results suggest a mechanism by which binding of the gp41 cytoplasmic tail to Gag within immature HIV-1 particles inhibits Env conformational changes on the surface of the virion that are required for proper viral membrane fusion with target cells [12]. This "inside-out" regulation of HIV

-1 fusion could play an important role in the virus life cycle by preventing the entry of immature, noninfectious particles [12].

Lastly, viral budding is completed by the membrane fission that is mediated by the p6 protein from Gag. The p6 protein binds to a protein that is normally involved in vesicular trafficking within cells called Tsg101 [13-15]. The p6 protein of HIV interacts with Tsg101 via its PTAP motif (Pro-Thr-Ala-Pro) or "late domain", and it is believed that this interaction allows the virions to bud with the help of the ESCRT I complex (endosomal sorting complex required for transport I (II and III)) which coordinates the multi-vesicular body pathway that recycles endosomes during normal cellular metabolism [reviewed in [16-18]]. Thus, consistent with its role as an obligate intracellular parasite, HIV makes efficient use of common cellular processes for sorting endosomes to facilitate its release from infected cells. Once the immature virions are released from the cell surface, the viral protease reaches a critical mass inside the budded virions and increased proteolysis of Gag precursors causes the condensation and assembly of p24 CA proteins that results in the structural formation of the mature virion core. A diagram of the complete HIV-1 life cycle is shown in Figure 3.

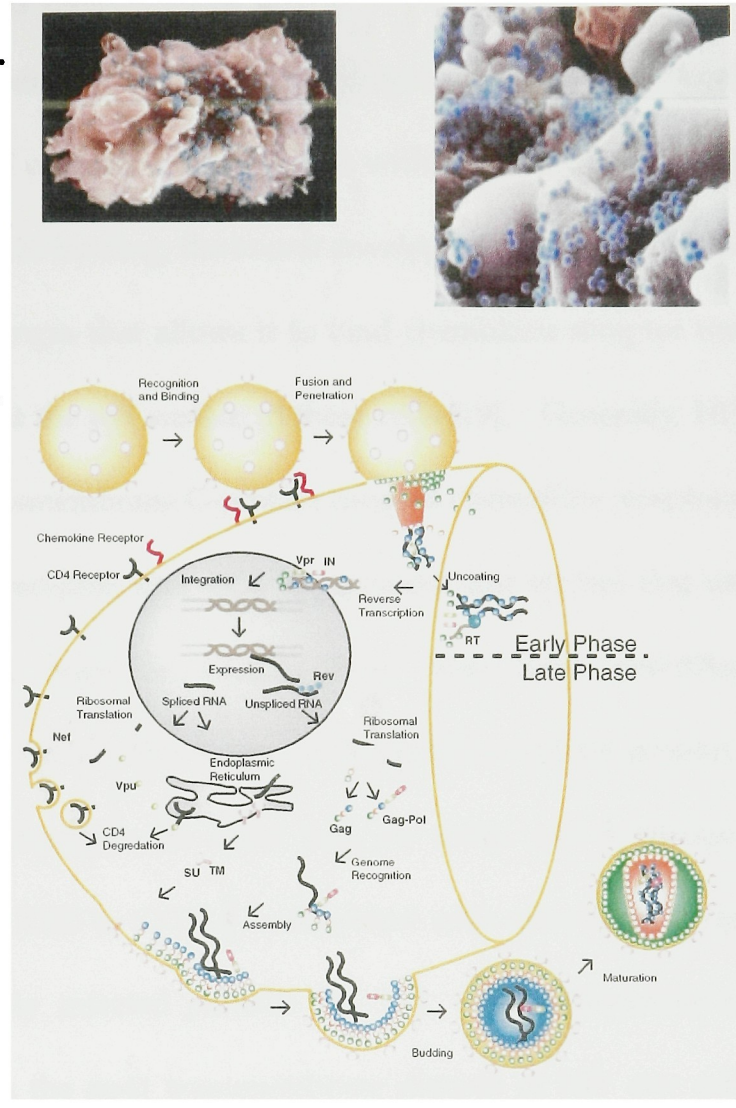


Figure 3. Overview of the HIV-1 life cycle. **A)** Electron micrographs of HIV virions (blue) attacking a CD4+ T cell (pink). **B)** A schematic diagram of HIV replication inside of an infected T cell is shown. Following attachment to the cell surface via CD4-gp120 binding/chemokine receptor engagement, envelope-mediated membrane fusion ensues. After fusion, the virion core enters the cytosol and undergoes a series of molecular interactions called "uncoating" and reverse transcription complexes (RTC) form allowing reverse transcription to occur. During or shortly after reverse transcription, RTC bind cellular proteins such as HMG I(Y) and become pre-integration complexes (PIC). PIC contain at a minimum IN, Vpr, and the viral DNA and are capable of traversing the NPC and entering the nucleus, where integration occurs. The "early phase" of the life cycle is complete after integration, then both full length and spliced viral RNA is transcribed, exported by Rev, translated by cellular machinery, and assembled at the plasma membrane facilitating the budding of new infectious virions. [Adapted from reference 113].

III. Regulation of the early events in the life cycle of HIV-1

As infection commences, HIV virions are drawn to the CD4+ molecules that adorn the surface of T cells via the interaction with the CD4 binding domain of gp120. After CD4-gp120 binding, the bound envelope protein undergoes a series of conformational changes that allows it to bind chemokine receptor molecules that are also present at the cell surface in these cells [19]. Generally, HIV relies on either of two 7-transmembrane G-protein coupled chemokine receptors, CCR5 or CXCR4, as the co-receptor after CD4 engagement, but viruses that use other chemokine receptors, such as CCR2b, have also been described [20]. Determinants within the variable loops of the gp120 envelope protein dictate which chemokine co-receptor the virus uses for entry, and a change in co-receptor usage from CCR5 to CXCR4 is associated with more rapid progression to AIDS in chronically infected patients [20, 21]. Once chemokine receptor binding has occurred, the gp41 transmembrane domain of HIV env achieves a fusogenic state, which is marked by its insertion into the cell membrane and the "zippering" of so called heptad repeat motifs that serve to bring the viral and cell membranes into close proximity until membrane fusion occurs [22, 23].

After fusion of the viral and cell membrane has occurred, the HIV core consisting of the p24 CA protein, the diploid viral RNA coated with the p9 NC protein, along with the viral enzymes RT and IN, p6 and Vpr, enters into the

cytosol and a mysterious series of molecular events known as "uncoating" ensues. The process of HIV-1 fusion and uncoating is depicted in Figure 4 below.

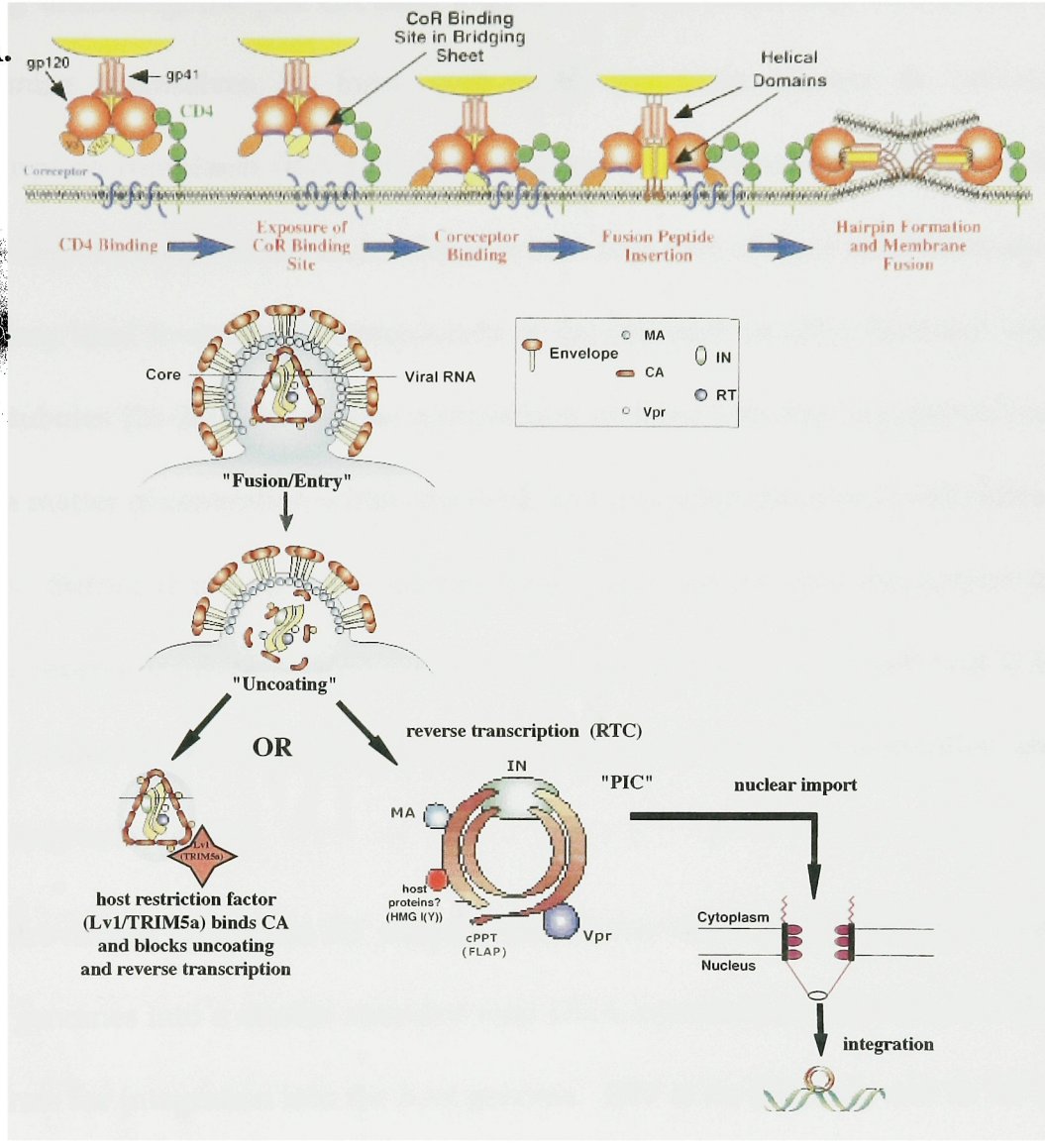


Figure 4. HIV gp-120-CD4/co-receptor binding, fusion/entry, uncoating and PIC formation. A) A schematic diagram of HIV envelope (gp120/gp41) protein binding CD4 on the surface of target cells, then exposure of the co-receptor binding site, binding to co-receptor (CCR5 or CXCR4), fusion peptide insertion into cell membrane, zippering of heptad repeats, and resulting membrane fusion. **B)** Depiction of the early events of the HIV life cycle immediately following viral fusion/entry. Upon entering the cytoplasm of the infected cell, the virion core disassembles during "uncoating", presumably unless blocked by the binding of host restriction factors (Lv1/TRIM5alpha) to the CA. Once uncoating is finished, the virion reassembles into macromolecular structures for reverse transcription (RTC) and nuclear import (PIC). These large sub-viral structures likely containing host cell proteins (such as HMG I(Y)) and interact with cellular nuclear import machinery in an unknown manner to cross the nuclear pore complex and integrate into the host chromosomal DNA. [Adapted from ref 241].

During uncoating, the p24 CA shell unravels and the remaining viral proteins reorganize themselves to form specialized structures known as reverse transcription complexes (RTCs) and pre-integration complexes (PICs) [reviewed in [24-26]]. Certain cellular molecules may also be a part of these complexes and they may bind to structural components of the cell such as actin filaments and microtubules [26-28]. The precise composition of these sub-viral complexes has been a matter of contention within the field, and a detailed discussion will follow below. Suffice it to say, these intermediate complexes provide the context in which reverse transcription occurs in the cytosol of the infected cell and it is widely believed that the processes of uncoating and reverse transcription are intricately coordinated.

All retroviruses specify an RT enzyme that allows them to convert their viral RNA genomes into a double stranded viral DNA intermediate that serves as the substrate for integration into the host genome. HIV is no different, and its RT is an RNA- and DNA-dependent DNA polymerase that makes a cDNA copy of the plus-stranded RNA genome from the incoming virion, then uses this cDNA as a template for second-strand synthesis to make the full length double stranded viral DNA ready for integration. The first step of HIV reverse transcription begins when a lysine transfer RNA (tRNA) molecule binds to a stretch of sequence near the 5′ end of the genome known as the primer binding site (PBS)

[reviewed in [29-33]]. From this primer, RT synthesizes a short stretch of DNA, while degrading the copied RNA in the newly-formed RNA-DNA hybrid. Next, by virtue of direct repeat regions located at each end of the viral RNA, this short stretch of newly synthesized cDNA can shift its hybridization from the 5′ end to the 3′ end of the genomic RNA in a process known as "strand switching" or "strand transfer." This allows the synthesis of a full-length minus strand and, in the process, initiates the generation of the long-terminal repeats (LTR). There are polypurine-rich regions (PPT) encoded within the genomic RNA that are resistant to the RNase-H activity of reverse transcriptase. These regions prime plus-strand DNA synthesis on the nascent minus-strand, and the central PPT has been implicated in the enhancement of viral nuclear transport [34]. A second strand-transfer is required to complete DNA synthesis. Successful initiation of reverse transcription requires both the correct positioning of the amino-lysyl-tRNA primer at the PBS, as well as the relaxation of the secondary structure of the viral RNA. The accessory protein Vif and the viral nucleocapsid (NC) assist in the completion of these two vital processes [7, 35].

When reverse transcription has been completed, the viral genome has to navigate through the viscous cytoplasmic milieu of the cell and somehow translocate across the nuclear pore into the nucleus in order to access the host cell chromatin, where integration subsequently occurs. As the viral genome and the

estimated size of the sub-viral reverse transcription and pre-integration complexes exceeds the known 50 kD (25 nm) exclusion limit of the mammalian cell nuclear pore [25, 36], it is unlikely that HIV nuclear import occurs by passive diffusion. Indeed, the precise composition of the sub-viral complexes that HIV forms once it enters the cytoplasm has been a topic of great contention and the process of HIV nuclear import remains ill-understood. As nuclear import is a key step in the early events of the viral life cycle and a major topic of this thesis, the composition of these sub-viral complexes and the proposed karyophilic properties of the individual viral proteins will be discussed in more detail in the sections to follow. After the viral DNA successfully localizes to the nucleus, it targets chromosomal DNA where the viral IN enzyme catalyzes the insertion of the HIV-1 genome into that of the host cell [37]. While it is widely held that retroviral integration occurs at random spots on the host genomic DNA, recent reports have suggested that HIV-1 displays a preference to insert its genome into actively transcribed genes [reviewed in [38]]. The strand transfer reaction catalyzed by the HIV IN allows the ends of the reverse transcribed viral DNA, which have had two nucleotides removed by the enzyme's 3′ processing activity, to effect a nucleophilic attack of the chromosomal DNA, creating a gapped (at each end) partially integrated intermediate. This gapped integration

intermediate is then filled in by host DNA repair enzymes [39], and the fully integrated retroviral DNA is called the provirus.

Once in the nucleus, an alternative fate of the viral DNA is also possible that allows for the covalent fusing of the viral DNA ends by enzymes involved in the non-homologous DNA end joining pathway, resulting in the circularization of the HIV genome [40]. These covalently closed circular viral DNA intermediates are called 1- and 2-LTR circles, and they were previously thought to be labile byproducts of failed HIV integration attempts. However, these viral episomes have unusual properties, such as their longevity inside of infected cells and their ability to express limited quantities of certain early viral genes, and Chapter 4 of this thesis highlights their potential use in gene therapy settings. While these 2-LTR circles appear capable of expressing small quantities of HIV Tat and Nef [reviewed in [41] and [42]], they are unable to sustain a productive infection in HIV permissive cells and they do not cause overt toxicity.

Although unintegrated viral DNA templates are capable of driving limited viral gene expression, the vast majority of HIV protein expression following integration and formation of the proviral state is the result of active transcription from the integrated viral DNA template. As the topic of this thesis involves the early events of the viral life cycle that precede the transcription of viral genes, these steps of the viral life cycle will be discussed briefly below. In the integrated

HIV template, the 5'LTR serves as the major promoter-enhancer for viral gene expression. The 5' LTR sequence possesses several regions (also present in the 3' LTR) that are recognized by common cellular transcription factors such as NFκB, Sp1, NFAT-1, and Ap-1 as well as a defined TATAA box. The defined unit that serves as the 5'LTR promoter includes a trio of binding sites for the constitutively expressed transcription factor Sp1, followed by the TATAA box and the transcription initiator element. Despite the presence of these transcriptional elements, the 5'LTR is in general a weak promoter of viral gene expression on its own [reviewed in [43]]. However, the presence of the transactivator protein (Tat), and its subsequent binding to the transactivation response (TAR) element, a 59-nucleotide stem-loop structure specified by the R region of the LTR [44], dramatically increases the effectiveness of the 5'LTR promoter, and viral gene expression following Tat-TAR complex formation increases one thousand-fold or more [45]. The Tat-TAR complex acts in concert with the cellular factor cyclin T, which recruits the cyclin-dependent kinase CDK9 to assemble the positive elongation factor b (pTEF-b) complex at the transcriptional start site of the viral template [46]. Recruitment of the pTEF-b containing the CDK9 kinase results in the hyper-phosphorylation of RNA polymerase II, allowing it to firmly attach and stimulate transcriptional elongation to form the viral RNA transcripts. As is the case for the transcription factors NFκB and NFAT-1, both cyclin T expression

and pTEF-b complex activity are up-regulated in activated T cells [reviewed in [47]] , reinforcing the notion that HIV enjoys a transcriptional advantage in these cells which may at least in part explain the virus' selection of this compartment as the major site for its replication.

Both unspliced and multiply-spliced viral RNAs are transcribed from the integrated provirus. As the cell tends to retain incompletely-spliced, intron-containing mRNAs in the nucleus, HIV developed a system to ensure that its full length unspliced genomic RNA and partially-spliced RNA transcripts can make it to the cytosol. The viral Rev protein binds to a cis-acting RNA element called the Rev-response element (RRE) that interacts with the cell's nuclear export machinery to shuttle unspliced or partially spliced viral RNA out of the nucleus and into the cytoplasm [48]. Rev itself is imported to the nucleus from the cytoplasm via direct interaction of its nuclear localization signal with the soluble nuclear import receptor karyopherin β [49]. As Rev and the unspliced RRE-bearing viral RNA accumulates in the nucleus, the nuclear export karyopherin, Crm1 (chromosome region maintenance protein 1) attaches to the Rev-RRE-RNA complex along with RanGTP and actively transports the viral RNA through the nuclear pore complex where it gains access to the cytosol. Once in the cytosol, the cellular protein RanGAP1 stimulates the hydrolysis of the attached RanGTP to RanGDP, and the exported viral RNA is released into the cytoplasm, where it

is free to attach to the Gag proteins that accumulate on the cytoplasmic side of the cell's plasma membrane during virion assembly as described above.

Composition and Karyophilic Properties of the HIV Pre-Integration Complex (PIC)

The nucleus is the largest membrane bounded compartment within eukaryotic cells, and a substantial amount of cellular energy is devoted to the shuttling of protein and ribonucleoprotein complexes through the nuclear pore complex (NPC)—the negotiable gate between the nuclear cytoplasmic compartments. After fusion and entry of its target cells, the Human Immunodeficiency Virus (HIV-1) must navigate its genome through the cytosol to the NPC and then into the nucleus where integration into the host's chromosomal DNA subsequently occurs [50, 51]. Although this property is essential to the virus' ability to infect its target cells, neither the precise mechanism of its nuclear import nor the identity of the cellular factors and viral determinants that are essential for it have been deciphered. For HIV-1, the study of this process is not only of immense academic interest, but also of significant medical relevance, as its clarification could lead to new therapeutic targets and novel treatments for HIV disease.

It has long been known that the viral genome is contained in a large nucleoprotein complex during reverse transcription of the viral RNA into its

cognate DNA copy—a process that appears to be complete before viral nuclear entry. This assembly of viral and host constituents is operationally defined as the pre-integration complex, or PIC [26]. The PIC is capable of traversing the cytoplasm, entering the nucleus, and targeting host chromatin. Once in the nucleus, the viral integrase protein (IN), assisted by the action of host enzymes, catalyzes the insertion of viral DNA into that of the host and subsequent repair of the chromosomal interruptions created by the integration event.

The PIC, and the reverse transcription complexes that precede it in the viral life cycle, likely constitute a series of structural and functional intermediates with changing composition. In fact, a variety of nucleoprotein complexes having different sedimentation velocities have been isolated from the cytoplasm and nucleus at specific times post-infection [52]. Not surprisingly, attempts to characterize the viral and cellular protein components of PICs using biochemical approaches have yielded disparate results. Integrase is consistently found as a constituent of the PIC, as would be expected for a viral protein that performs obligate catalyses on the viral DNA substrate in both the cytoplasm and nucleus. Other PIC-associated viral proteins include matrix (MA), Vpr, and, less frequently reported, nucleocapsid, capsid, and reverse transcriptase (RT) [26, 36, 53, 54].

A facilitating role in the nuclear import of the viral genome has been attributed to a number of PIC components, including MA, Vpr, IN, and elements in the viral DNA itself. Nevertheless, assignment of the relative importance of each remains controversial. While some studies have implicated MA as a mediator of infection in non-dividing cells [55, 56], others have shown that infection with viruses that are altered in MA proceeds efficiently in the absence of either proposed karyophilic determinants within MA [57-59] or the majority of the MA protein [60]. In addition, while the ability of Vpr to interact with the nuclear pore complex has been well documented [61, 62], and varying degrees of block to viral replication have been observed in its absence [63, 64], virions lacking both Vpr and the proposed NLS regions in MA can still efficiently infect non-dividing cells [54, 59]. Recent studies using confocal and electron microscopy to visualize the fine structure of HIV reverse transcription complexes (RTCs), a conglomerate that is believed to be a substructure of the PIC, confirmed the presences of Vpr and IN within RTCs at 3 to four hours post infection with VSV-pseudotyped HIV vectors [25].

A role in PIC nuclear import has also been attributed to the central DNA flap—a discontinuity of the plus-strand of the HIV genome consisting of a 99 basepair region of triple helix viral DNA, which apparently acts *in cis* to enhance nuclear targeting of the incoming PIC [65]. Viruses lacking this central DNA flap

are not compromised for reverse transcription, but exhibit a slight 2-5 fold defect in nuclear import [34]. Addition of the central DNA flap synthesis in lentiviral vectors that normally lack the structure increases their ability to transduce a variety of cell and tissue types *in vitro* [34] and *in vivo* [66]. However, recent experiments suggest viruses lacking the central DNA flap exhibit near wild-type levels of nuclear uptake under a variety of experimental conditions, and the effects of this viral structure on nuclear import may be cell type-specific and less significant than originally thought [67, 68].

The final PIC component with reported karyophilic activity is integrase. Although an initial study characterized the intracellular localization of an IN-β-galactosidase fusion protein as cytoplasmic [69], further work using both transient transfection and *in vitro* nuclear import assays has shown that IN localizes predominantly to the nucleus [70-73]. One study reported that the mutations K186Q and Q214ŁQ216L prevent the import of isolated IN or of other PIC-associated proteins (and presumably of the PIC itself) in assays performed *in vitro* [54], however, others have observed wild-type accumulation of nuclear forms of viral DNA in the presence of these mutations [70, 74-76]. In a more recent study, a peptide spanning amino acids 161-173 of IN was reported to act as a transferable NLS. Mutations at residues V165 and R166 abrogated the nuclear localization of an IN-pyruvate kinase fusion protein and, in the context of

infection, impaired the accumulation of viral DNA in the nuclei of both dividing and non-dividing cells [77]. Again, the interpretation of these results has been challenged [78] and reassessed, and it was discovered that the V165 and R166 mutations have pleiotropic effects on the early viral life cycle (generally at the step of reverse transcription) which overshadow their previously described defects in nuclear import [67]. Thus, the precise mechanism of HIV nuclear entry, as well as the identity of the cellular factors and viral determinants that are essential for it, remains elusive.

Cellular proteins that have been associated with the PIC include the high mobility group protein HMG I(Y) and another protein called barrier to auto-integration factor 1 (BAF). HMG I(Y) is believed to bind to the reverse transcribed viral DNA contained in the PICs and may facilitate the histone-like winding and "coiling" of the PIC DNA, reducing its size from the approximated 3.3 microns if it were unwound, to the 56 nm estimated diameter of the PIC [reviewed in [28, 79]. The BAF protein is thought to prevent the viral DNA from integrating into itself in an intramolecular fashion and can enhance the catalytic activity of salt-stripped PICs *in vitro*, though it is unclear whether this molecule is directly associated with the incoming PIC and its relative importance to the viral life cycle is unknown[80]. Other proteins reported to associate with the incoming PIC include chromatin remodeling proteins such as SNF5 (INI-1) and PML [81] .

Nuclear Import Overview

I. Structural features and functional relevance of the nuclear pore complex (NPC)

The mammalian cell nucleus is a key adaptation that marks the evolutionary divergence of eukaryotic cells from lower level organisms. Accordingly, a substantial amount of cellular energy is devoted to the shuttling of protein and ribonucleoprotein complexes into and out of the nucleus, through the nuclear pore complex (NPC)—the negotiable gate between the nuclear and cytoplasmic compartments. While the nucleus appears to be a bounded compartment under the light microscope, under the electron microscope it appears dotted with numerous holes, which are the nuclear pore channels. An electron micrograph of a mammalian cell nucleus is shown in Figure 5 along with a higher magnification image of nuclear pores from an amoeba (*dictyostelium discoideum*). The outer nuclear membrane is contiguous with the endoplasmic recticulum (ER) membrane, while the inner nuclear membrane encapsulates the host cell chromatin. The points at which these two membranes meet and interconnect are the junctions where nuclear pores form [shown in Figure 6], giving rise to the avenues of molecular transport between the cytoplasm and the nucleoplasm that the NPC governs.

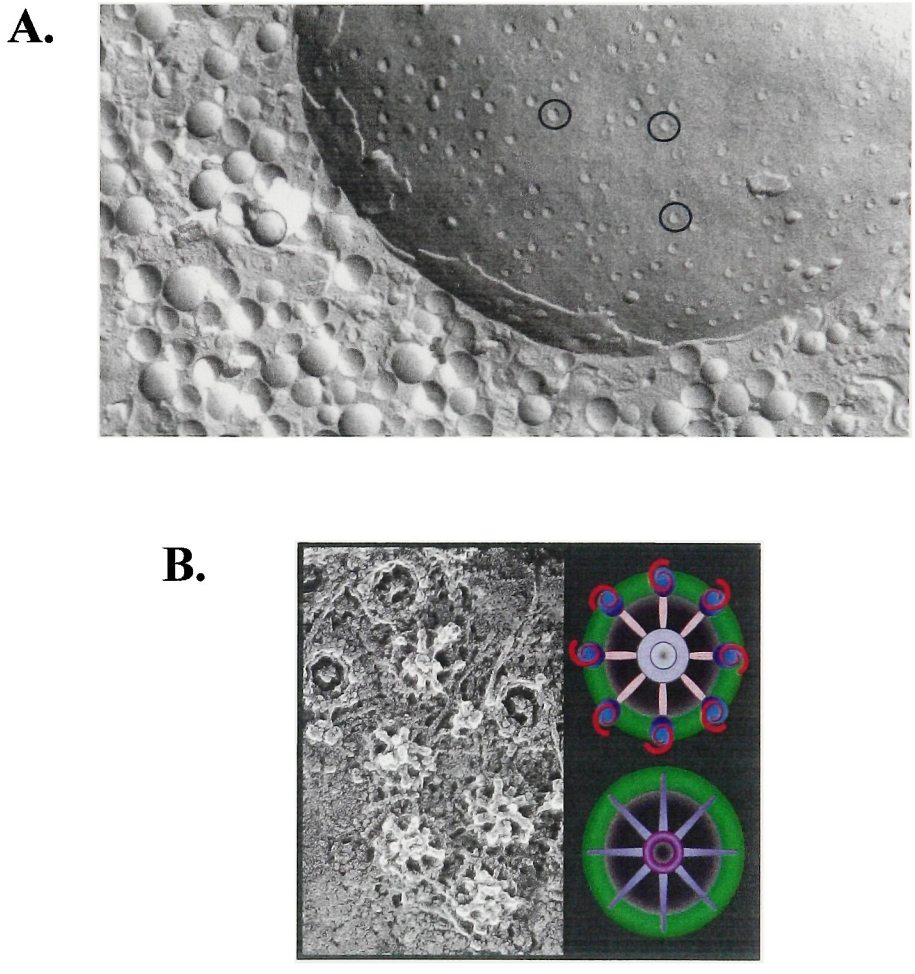


Figure 5. Electron micrographs of the fine structure of the vertebrate NPC. A) Cryo-electron micrograph of a mammalian cell with visible nuclear pores (black circles). **B)** Electron micrograph NPC from a soil-dwelling amoeba, Dictyoselium discoideum, with views from both the cytoplasmic and nuclear side of the NPC indicated (diagram). The eight-fold symmetry of the NPC is clearly apparent. [Adapted from ref 242].

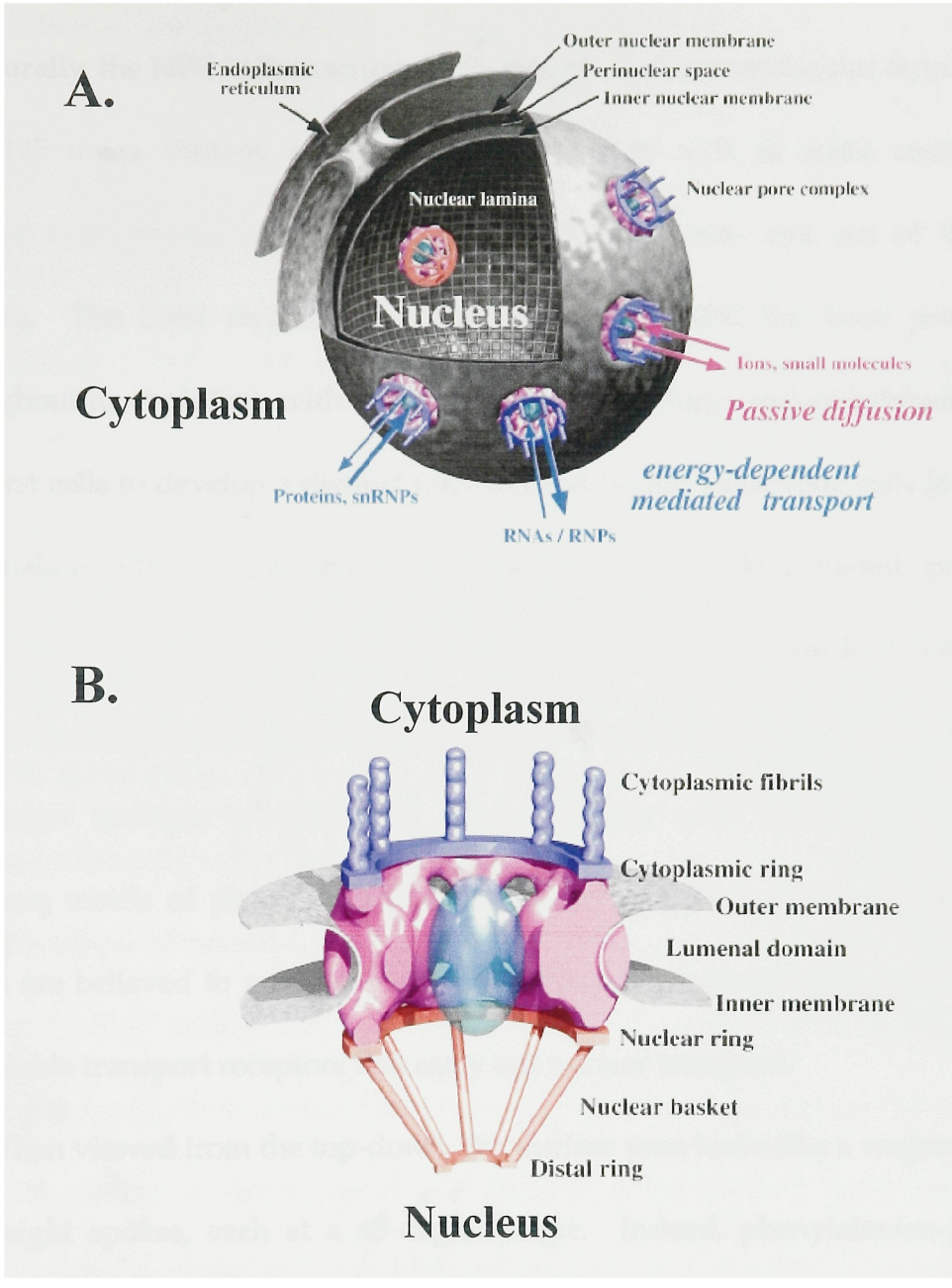


Figure 6. Diagram of the Nuclear Pore Complex (NPC). A) The NPC allows passive diffusion of ions and small molecules. Nuclear proteins, RNAs, and ribonucleoprotein (RNP) particles larger than ~9nm are selectively and actively transported through NPCs by a signal-mediated and energy-dependent mechanism called nuclear import. **B)** Surface renderings of the nuclear pore complexes revealing the central framework, which exhibits strong 8-fold symmetry and thus consists of two identical halves relative to the central plane of the nuclear envelope. [Adapted from references 43 and 44].

Structurally, the NPC is immaculate in its design—a macromolecular structure of over 125 mega Daltons composed of constant as well as static constituent proteins that interact dynamically to govern traffic into and out of the cell nucleus. The basic structure and function of the NPC has been preserved throughout its evolution, with a high degree of homology evident between yeast (the first cells to develop a distinct nucleus) and higher eukaryotic cells [82]. The mammalian NPC is comprised of a set of at least 30 different proteins, collectively termed nucleoporins [83]. The NPC displays eight-fold rotational symmetry, therefore every nucleoporin is present at a copy number of either 8 or an integer multiple of 8 [82, 84]. These proteins often contain amino acid sequence motifs of phenylalanine and glycine dipeptide repeats (FG repeats), which are believed to mediate the main interactions between nucleoporins and the soluble transport receptors that carry out nuclear transport.

When viewed from the top-down, the nuclear pore looks like a wagon wheel with eight spokes, each at a 45-degree angle. Indeed, phenylalanine-glycine dipeptide repeats or variations thereof (i.e. GLFG or FXFG) are present in nearly half of all of the nucleoporins identified, leading to the commonly used nomenclature for them as "FG nups." These FG nups come in numerous shapes and sizes. Some are long, filamentous proteins with multiple domains and enzymatic activity, such as the cytosolic Nup358. Others line the core of the

central channel and are more compact, such as Nup62. Regardless of their shape or size, the FG nups that comprise the NPC are all oriented with their FG repeats facing the interior of the nuclear pore channel, forming a pathway of FG repeats for soluble nuclear import factors, such as the karyopherins (also called importins), to interact with as they transport their cargo across the nuclear pore. The precise mechanism for FG nup-mediated nuclear translocation is unknown, however several models have recently been proposed to explain this phenomenon. Nuclear translocation occurs against a concentration gradient, and kinetic studies have indicated that a single NPC can accommodate a mass flow of up to 80 million Daltons per second, corresponding to about 1000 translocation events per second per NPC [reviewed in [85]].

One model of nuclear translocation that has been proposed is the "Brownian Affinity Gating" model [86]. This model suggests that a given polypeptide moving in a rapid and random fashion through the cytolsol must pay an entropic price for gaining access to the central channel of the nuclear pore, and, once there, remains in a 'transition state' for nuclear translocation [86]. The larger the protein, the higher the entropic penalty for its gaining access to this transition state, and the lower its probability to passively diffuse through the NPC. For large proteins, active transport across the NPC is mediated by the interactions of the FG nups (which line the interior of the NPC and stretch from the cytosolic to

the nuclear compartments of the pore) with soluble transport factors that bind to NLS containing large proteins, acting in a concerted 'enzymatic' fashion to help the large proteins overcome the entropic barrier to nuclear translocation.

Another model is the hydrophobic exclusion or "selective gate" model [87]. This model suggest that the FG nups which line the central channel of the nuclear pore create a hydrophobic environment that acts as a barrier to keep large, hydrophilic proteins out. Macromolecules containing NLS sequences, and therefore bound by soluble import factors such as the karyopherins would be chaperoned across the pore by locally disrupting the hydrophobic "gate", creating a selective partition for the molecule to pass through [87, 88] . A major difference between the Brownian affinity gating and the hydrophobic selective gate theories is that the latter calls for weak interactions between the FG repeats themselves, while the former suggest that the FG repeats bind only to the karyopherin-bound cargo proteins that are being imported. In yet another proposed scenario, called the affinity gradient model, the FG nups are assembled as an array of interaction sites with progressively increasing affinities from the cytoplasmic to the nuclear sides of the NPC [89]. In this model, selective nuclear transport is ensured because non-karyopherin-bound cargo proteins would be unable to interact with FG nups and therefore would not undergo the cycles of association/dissociation with the nucleoporins lining the central channel and

would not be transported across the NPC. A shematic diagram of these proposed models of nuclear-cytoplasmic transport is shown in Figure 7.

II. Soluble transport receptors and the Ran GTPase system

For molecules that are too large to passively diffuse through the NPC, nuclear-cytoplasmic transport begins with the recognition and binding of such proteins by soluble transport receptors in the cytosol. Once these protein 'cargos' are bound by soluble transport receptors, the entire complex localizes to the nuclear compartment where it docks at the NPC [reviewed in [90, 91]]. These soluble transport receptors are called karyopherins (also referred to as importins, exportins and transportins) and they have the unique ability to bind to protein cargos that display a specific amino acid sequence, known as a nuclear localization signal (NLS) or nuclear export signal (NES), that determines the directionality of their transport through the NPC. Fourteen different karyopherins have been identified in the yeast *S. cerevisiae*, and at least 20 are estimated to exist in vertebrates, each with the potential to recognize a different NLS or NES [reviewed in [92]]. In addition, these karyopherins often exist in α and β isoforms, which form any number of different heterodimeric complexes capable of binding to different FG nucleoporins in the NPC.

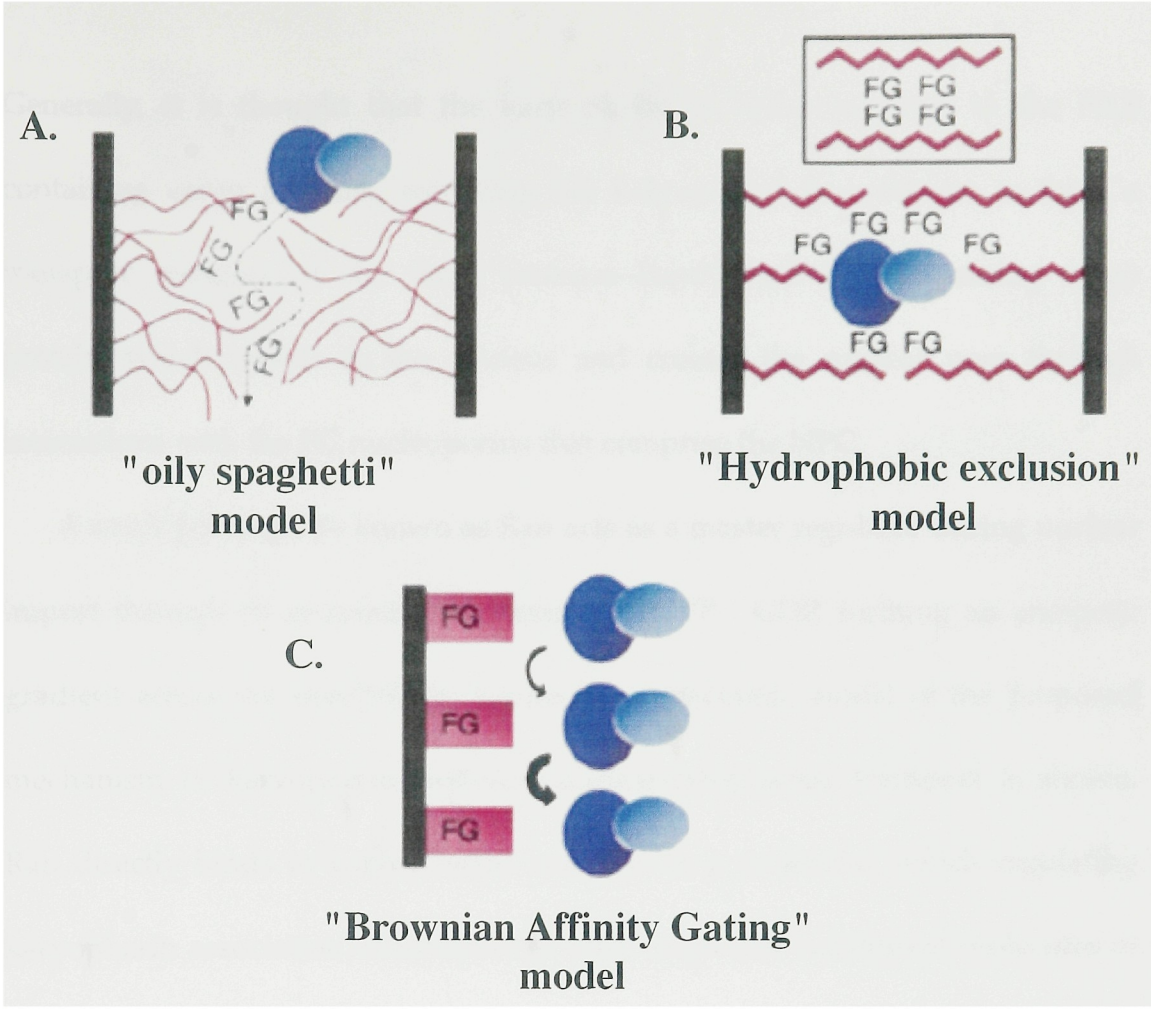


Figure 7. Models of nuclear-cytoplasmic transport across the NPC. Schematic diagrams of the three leading proposed models for how nuclear transport occurs across the NPC are shown. **A)** The "oily spaghetti" model suggests that a karyopherin-cargo protein complex slides through a network of filamentous FG nucleoporins that normally excludes macromolecules that do not directly interact. These interactions may facilitate random movement of the karyopherin-cargo protein complex through the network of FG nups and ultimately across the NPC. **B)** The "hydrophobic exclusion" model proposes that the FG nups lining the central pore support weak hydrophobic interactions between one another that creates a barrier of hydrophobicity that non-karyopherin bound proteins will be unable to cross. **C)** The "Brownian motion/Affinity gating" model suggests that the FG nups may provide a series of sequential binding sites of increasing affinity from the exterior to the interior of the NPC to which the translocating karyopherin-cargo protein complexes temporally attach. FG binding sites at the NPC faces create a high concentration of transport complexes near the NPC, increasing the probability that the translocating protein complexes will move across it by random diffusion. [Adapted from reference 112].

Generally, it is thought that the karyopherin α molecules bind to the NLS containing cargo proteins, recruiting the karyopherin β molecules to form a transport complex of the three proteins--kapα/kapβ+NLS-containing cargo protein--which travels to the nucleus and crosses the nuclear pore through interactions with the FG nucleoporins that comprise the NPC.

A small polypeptide known as Ran acts as a master regulator during nuclear import through its enzymatic conversion of GTP GDP, forming an energetic gradient across the pore[93]. In Figure 8a, a schematic model of the proposed mechanism of karyopherin-mediated nuclear-cytoplasmic transport is shown. Ran directly binds to karyopherins and acts as a molecular switch regulating karyopherin association/disassociation with cargo and constituent molecules of the NPC [reviewed in [90]].

During import, RanGTP binding to NLS-karyopherin complexes triggers cargo release in the nucleus, whereas for export, RanGTP stabilizes the karyopherin-cargo interaction, allowing the complex to exit across through the NPC [92]. A steep RanGTP/RanGDP gradient has been measured across the nuclear envelope and is key for dictating the directionality of transport [94]. This gradient is maintained by several essential Ran co-factors, such as RanGAP1, RCC1, and NTF2.

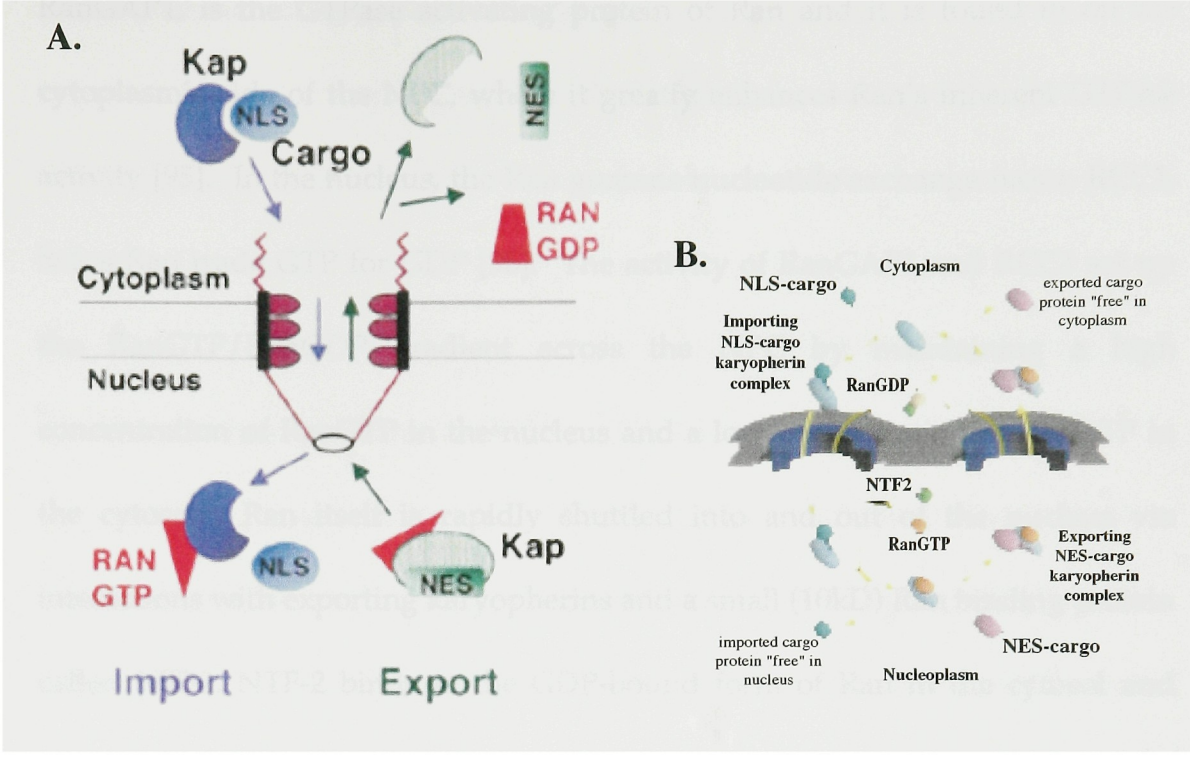


Figure 8. Soluble transport receptors and the RanGTPase system. Nuclear import is carried out by soluble nuclear translocating receptors called karyopherins (also called importins or exportins) in conjunction with an energetic gradient of GTP hydrolysis catalyzed byt the small GTPase Ran. **A)** A schematic diagram of Ran-mediated karyopherin-assisted nuclear import is shown. A given cargo protein (light blue) bearing a functional NLS is bound by a complex of karyopherin alpha and beta (dark blue). The cargo protein complex is then shuttled through the central channel of the nuclear pore complex via the interactions of the bound karyopherins with the FG nucleoporins (purple) that comprise the NPC. Once inside the nucleus, the karyopherin-bound cargo protein binds to RanGTP (red triangle) in the nucleus causing it to be released into the nucleus and allowing the unbound karyopherin complex to be recycled to the cytosol after RanGAP-mediated RanGTPRanGDP hydrolysis. **B)** RanGDP (pink) is then recycled to the nucleus by a molecule called NTF2 and is reloaded with GTP by RanGEF to begin another import cycle. [Adapted reference 111].

RanGAP1, is the GTPase activating protein of Ran and it is found in on the cytoplasmic side of the NPC, where it greatly enhances Ran's inherent GTPase activity [95]. In the nucleus, the Ran guanine nucleotide exchange factor, RCC1, helps Ran trade GTP for GDP [96]. The activity of RanGAP1 and RCC1 set up the RanGTP/RanGDP gradient across the NPC by maintaining a high concentration of RanGTP in the nucleus and a low concentration of RanGTP in the cytosol. Ran itself is rapidly shuttled into and out of the nucleus via interactions with exporting karyopherins and a small (10kD) Ran binding protein called NTF2. NTF-2 binds to the GDP-bound form of Ran in the cytosol and relocalizes it to the nucleus through its interactions with the FG nups that line the NPC. RCC1 is associated with nuclear chromatin, and RCC1 mediated GTP GDP nucleotide exchange in this compartment causes the dissociation of NTF2-RanGDP, freeing up more Ran to bind GTP in the nucleus. While Ran is small enough (24kD) to passively diffuse across the NPC into the nucleus, NTF2-assisted active transport of RanGDP enhances its import efficiency and ensures the proper distribution of Ran between the cytoplasm and nucleus [97]. For larger cargo protein cargos, there may be additional requirements for the RanGTPase system [98].

While Ran-independent mechanisms for NPC transport have been suggested (for example, during certain types of RNA export), it is widely accepted that the

majority of nuclear-cyctoplasmic transport of large molecules fufill the energetic requirements for transport via RanGTP hydrolysis. A diagram of Ran-GTP recycling and how it intersects with karyopherin-mediated nuclear transport is shown in Figure 8b.

III. Nuclear import of mammalian RNA and DNA viruses

All DNA viruses and many RNA viruses that infect mammalian cells must localize their genomes to the nucleus in order to establish a productive infection. HIV-1, the virus that is the subject of this thesis, is no different in this regard.. Therefore, in order to provide a context for the studies on HIV-1 nuclear import described herein, a brief review of known mechanisms of nuclear import for several other viruses is provided below.

Adenoviruses nuclear import

The Adenovirus family of viruses are non-enveloped DNA viruses with characteristic isometric capsids of 60–90 nm in diameter and a linear double-stranded DNA genome. These complex and functionally sophisticated viruses generally encode 11–15 different structural proteins that carryout the various duties required to establish persistent infection of target cells [reviewed in [99]]. Virion proteins include receptor-binding fibers extending from specialized

penton base complexes at the vertices of the icosahedral cores, and several DNA binding proteins such as the covalently bound p55 terminal protein at the 5′ ends of the viral DNA [100-103].

After binding to cell surface CAR receptors, these viruses are internalized by receptor-mediated endocytosis [104]. Following endocytosis, they penetrate the endosomes by inducing acid-enhanced lysis of the endosomal-limiting membrane, and are then released into the cytoplasm [105]. The viruses are then transported through the cytosol to the nucleus where a large fraction bind to the nuclear pore complexes that adorn the nuclear membrane [106]. During nuclear transport, adenoviruses particles use microtubules and microtubule-dependent motors [107].

As the adenovirus particles migrate from the endosomes to the NPC, they undergo a series of programmed steps that appear to weaken the integrity of the sturdy viral capsid, allowing it to release its precious cargo of viral genetic information only once it has localized to the nuclear pore. The molecular alterations are triggered by receptor interactions, exposure to acidic pH, re-entry into a reducing milieu, and finally by interactions with the NPC [reviewed in [108]]. As fibers are stripped from the incoming viral particle once it emerges from the endosome, capsid stabilizing proteins dissociate from the viral core, and a protein that connects the DNA to the inside capsid's wall, protein IV, is

digested by a viral cysteine protease called p23 [109, 110]. Once the stripped adenovirus particle reaches the NPC it encounters factors that trigger the final stages of particle dissociation. The FG nucleoporin Nup214/CAN is the docking site for adenoviral particles at the NPC, and unlike some other viruses, nuclear transport of the Ad2 virion presumably occurs in a karyopherin-independent manner [111]. By directly targeting a constituent of the NPC, in this case the filatmentous Nup214/CAN protein present on the cytoplasmic side of the NPC, the adenovirus particle ensures robust and rapid nuclear localization of its genome. Adenovirus has taken nuclear import a step further, and it appears that nuclear localization of the viral genome is a key regulatory step in the viral life cycle rather than a mundane requirement. This regulation is manifested in the binding of histone H1 to the Nup214/CAN docked adenovirus particle, which allows the viral DNA to be shuttle through the nuclear pore via the same karyopherin-dependent mechanisms that histones use during normal cellular metabolism [111].

Of the DNA-binding proteins encoded by adenovirus, protein VII is thought to have a histone-like DNA-condensing function. It enters the nucleus at the same time as the DNA, and it is possible histone H1 and protein VII act in concert to thread the DNA through the pore as a nucleoprotein complex [111, 112]. The terminal protein p55 covalently associated with the 5′ end of each Ad

DNA strand also enter the nucleus and play an important role in anchoring the viral DNA to the nuclear matrix [113]. Another Ad DNA binding protein, protein V, does not seem to enter with the DNA and its function during viral nuclear import remains unknown [108]. A small amount of the hexon protein from the incoming virus also finds its way into the nucleus, but this probably occurs independently of nuclear import of the viral DNA. While a precise role in the viral life cycle has not been assigned to all of the proteins encoded by adenovirus, the mechanism of Ad nuclear-cytoplasmic transport is one of the most clarified for mammalian viruses.

Hepatitis B virus nuclear import

The hepatitis B virus (HBV) is a prototype member of the family *hepadnaviridae*. It is enveloped, and has an icosahedral capsid core 32 nm in diameter. The protein shell is composed of 240 copies of a single protein, called the core protein, and expression of this protein alone is sufficient for the formation of a core particle [114]. Hepadnaviruses have a DNA genome, but because they replicate via an RNA intermediate, they require reverse transcription [115]. HBV and other hepadnaviruses enter the cell via receptor mediated endocytosis [116-118] and gain access to the cytosol via membrane fusion in endosomes [116]. The viral DNA is then transported to the nucleus

where the RNA template for reverse transcription (the pregenome) is synthesized by cellular RNA polymerase II [119]. After export to the cytoplasm, this viral RNA is used as a template for translation of the core protein and polymerase (polymerase or "P" protein), and it is also packaged into progeny core particles [120]. The RNA is reverse transcribed by the trapped P protein to the partially double-stranded DNA, and the polymerase remains covalently linked to the minus strand [121].

As the viral DNA in the incoming HBV virion is not intrinsically karyophilic [122], one or more of the structural proteins of the core must be responsible for mediating nuclear import of the HBV genome. The core protein is the leading candidate as a facilitator of viral nuclear import. The most compelling evidence that the core protein is essential to HBV nuclear import comes from studies with bacterially-expressed core particles that contain the core protein but no other components of the virus [123]. In an in vitro assay using digitonin-permeabilized human hepatoma HuH-7cells, these HBV cores bind specifically to the NPCs [123, 124]. NPC binding was inhibited by wheat germ agglutinin, by anti-nuclear pore complex antibodies, and by peptides corresponding either to classical nuclear localization signals (NLS) or to COOH-terminal sequences of the core protein [123]. Binding was dependent on the classical soluble nuclear transport receptors karyopherin α and β. Nuclear

localization only occurred when the core protein was phosphorylated, suggesting that phosphorylation is required to induce the exposure of a cryptic NLS in the C-terminus of the HBV core protein that allows the virion to bind karyopherins and traverse the NPC by co-opting classical cellular nuclear import pathways [123, 124]. Indeed, the C-terminus of the HBV core protein contains five potential Ser/Thr phosphorylation sites, and this arginine-rich stretch of sequence (amino acids 145–183 in HBV) has homology to classical basic nuclear localization signals [123, 125, 126].

The HBV NLS sequence overlaps with the RNA packaging domain (amino acids 141–164), as well as with a region that supports plus-strand DNA synthesis during genome maturation [127, 128]. Prior to core maturation, these C-terminal sequences are present inside the central cavity of the core [126]. However, to become functional NLSs, they must be somehow exposed at the outer surface of the core. Perhaps core protein phosphorylation induces a conformational change that exposes these peptides on the outer surface of the virion where they can serve as substrates for karyopherin-mediated nuclear import. Cryoelectron microscopy studies show large holes in the capsid wall between individual core proteins [114]. These openings could allow the NLS peptides to poke out to the virion surface, where they can have access to the nuclear transport receptors (kapα/β) in the cytosol, without major changes in overall core architecture.

Whether or not significant viral core disassembly is necessary for HBV virion nuclear import (as is seen in the case of adenovirus nuclear import) is uncertain. However, the recent report from Rabe et al. suggests that HBV capsids were imported in intact form through the nuclear pore and localized to the interior of the NPC at the nuclear basket [124]. Import depended on phosphorylation of the capsid protein and was mediated by the cellular transport receptors karyopherin-α and -β. However, the uncoating reaction was independent of RanGTP binding, and occurs through a mechanism different than the one normally used to dissociate karyopherin-imported cargo in the nucleus [124]. Hepadnavirses are unusual in that a fraction of the cores do not leave the cell in the form of progeny virus but deliver their DNA directly into the nucleus of the host cell in which they have assembled. This is apparently required to replicate the viral DNA in the nucleus, which eventually reaches 20–50 copies per cell, and may be responsible for sustaining a level of latency in infected cells [115].

Influenza nuclear import

Influenza viruses are enveloped animal viruses with a segmented, negative-sense RNA genome [129]. Individual influenza virions are typically 80 -120 nm in diameter and often appear filamentous, especially when taken from fresh isolates [129]. They have eight distinct RNA molecules (viral chromosomes)

individually packaged into viral ribonucleoprotein complexes (vRNPs) that contain multiple copies of nucleoprotein (NP) and a single copy of a heterotrimeric polymerase. NP forms a proteinaceous core around which the RNA is wound in a helix [130]. The unwound length of the individual vRNPs varies from 20 to 80 nm depending on the size of the RNA [131]. However, the diameter of the rod-shaped vRNP particles is constant (between 10 and 20 nm) and they are small enough to overcome the size exclusion limit (~25 nm) of the nuclear pore and passively diffuse through the NPC. Indeed, current evidence suggests that incoming influenza vRNPs enter the nucleus in intact form through the NPCs [132], reminiscent of HBV nuclear import of its mostly intact viral particle. The NP and the viral polymerase accompany the RNA into the nucleus where they are required for transcription and replication [131].

Prior to nuclear import of vRNPs, these sub-viral structures must undergo two distinct low-pH-induced changes. First, an acid-induced conformational change in HA is required to trigger fusion and escape of the vRNPs into the cytoplasm [133] . Acidification has also been shown to induce an irreversible change in M1, resulting in its dissociation from vRNPs [134, 135]. The dissociated vRNPs are thus delivered to the cytosolic compartment from which they rapidly and efficiently move into the nucleus via the NPCs. Similar to what is seen during adenovirus import, dissociation of the influenza sub-viral complexes is a

key regulatory event in its virus life cycle. It has been shown that the antiviral drug amantadine, which blocks the M2 ion channel, thereby preventing endosomal acidification and viral uncoating, also abrogates nuclear import of the vRNPs [136-138].

While all of the known protein components of the vRNPs carry some type of NLS (as they must undergo transport to the nucleus after synthesis in infected cells), responsibility for nuclear transport of the influenza genome appear to fall on the NP protein [139]. NP is thought to mediate influenza nuclear import via classical basic-type NLS and interaction with the heterodimeric karyopherinα/β molecules [139]. NP can also mediate the import of RNA with which it is complexed, implying that the NLS does not overlap with the RNA interaction domain. The import of the NP proteins alone was originally shown to depend on a basic-type NLS close to its N terminus [140, 141], but it is probable that this N-terminal NLS is dispensable for the process of viral genome nuclear import, as it is not conserved in other orthomyxovirus subtypes [142] .

Unlike adenovirus particles, there is no evidence that influenza vRNP transport along any cytoskeletal elements once they have been released from endosomes [143, 144].

Figure 9 summarizes the various routes of nuclear entry that are employed by adenovirus, hepatitis B virus, and HIV, which is the subject of this

thesis. All infectious viruses that rely on the nucleus as a site for their replication are presented with the challenge of passing through the nuclear pore complex (NPC) which tightly regulates molecular traffic into and out of the nucleus. Consistent with their roles as obligate intracellular parasites, many viruses have adapted to use the same machinery that the host cell utilizes for macromolecular transport through the NPC. Thus, the capsid proteins of several viruses, including those adenoviruses and hepadnaviruses like HBV, have evolved to contain NLS sequences that allow them to interact with soluble nuclear transport receptors like the karyopherins in order to deliver their genetic information to the nucleus. Notably, many such viruses undergo significant molecular alterations (i.e. decapsidation, stripping, acidic-induced structural changes) before they are capable of performing nuclear import. Lentiviruses like the human immunodeficiency virus (HIV) are no different in this regard, as a significant amount of rearrangement of the viral core constituents occurs as virions enter the target cell cytoplasm ande pre-integration complexes (PICs) are assembled. This thesis describes the potential role of one of the viral proteins that is present throughout the transmogrification of the incoming HIV viral particle, namely the HIV integrase, in facilitating the active nuclear transport of the reverse transcribed viral genome into the nucleus.

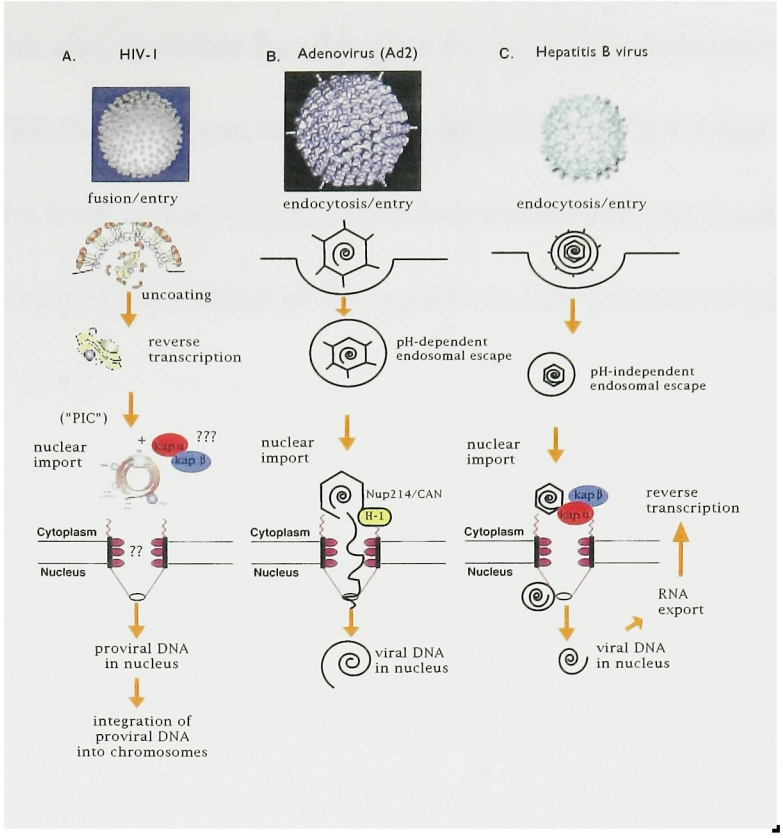


Figure 9. Mechanisms of DNA and RNA virus nuclear translocation. The proposed mechanisms for nuclear import for HIV-1, Adenovirus type-2 (Ad2), and Hepatitis B virus (HBV) are shown. **A)** HIV-1 nuclear import follows viral fusion/entry and reverse transcription and precedes the step of proviral DNA integration in the nucleus. It is unclear whether HIV-1 utilizes classical nuclear import receptors such as karyopherin-alpha and karyopherin-beta, and the precise mechanism of HIV-1 nuclear import is unknown. **B)** Ad2 nuclear import follows viral endocytosis/endosomal escape and relies upon a combination of host cell factors (karyopherin-alpha, karyopherin-beta, histone H1, and heat shock protein 70, all bound to the NPC via Nup214/CAN) to facilitate the breakdown of its capsid at the NPC, allowing the viral DNA to be injected through the nuclear pore and into the nucleus. **C)** HBV nuclear import occurs after the virus enters the cell by endocytosis and escapes the endocytic vesicle via a pH-independent mechanism resulting in their release into the cytosol. Once in the cytosol, HBV virions bind karyopherin-alpha/karyopherin-beta heterodimers, traffic to the nucleus, and disassemble at the NPC to release the covalently closed circular viral DNA into the nucleus where it is transcribed into viral RNA, exported, and then reverse transcribed to HBV viral DNA in the cytosol.

This work also describes the identification of certain endogenous host cell factors (Lv1/TRIM5-α) that can bind to incoming HIV and SIV virions, preventing them from completing reverse transcription and presumably abrogating their ability to localize to the nucleus in non-permissive primate cells.

CHAPTER 2. POST-ENTRY RESTRICTIONS TO HIV-1 REPLICATION

I. Species-specific restriction of lentiviral infections

Many lentiviruses that infect humans and non-human primates exhibit a highly restricted species tropism. HIV-1, for example, is unable to replicate in cells derived from several nonhuman primates, including rhesus macaques, the most prominent monkey species used in animal models of AIDS [145] . Therefore, it would be of great scientific interest to those seeking to develop better animals models to study HIV-1 to determine the precise step of the viral life cycle at which replication is blocked in such non-permissive primate cells. It had previously been demonstrated that the block to HIV-1 replication in macaques occurs primarily during the early steps of the viral life cycle, such that reverse transcription does not proceed to completion [146, 147]. However, the exact point in the viral life cycle where this species-specific restriction was manifest remained unknown. Indeed, the virus could have been inhibited at any of the steps that precede reverse transcription, such as envelope/receptor binding, viral membrane fusion/entry, or during the process of viral capsid uncoating.

Observation of the SHIV rhesus macaque animal model, where a simian immunodeficiency viruses from macaques (SIVmac) is engineered to contain

envelope genes from HIV-1, reveals that such hybrid viruses can replicate in these animals [148]. Therefore, inefficient HIV-1 replication in this non-permissive species cannot be explained entirely by envelope-receptor incongruities [149-151]. While some portion of the post-entry restriction of HIV-1 in primates may be due to surface receptor-envelope incompatibility [152], certain primate cells display dramatic differences in their ability to support the early steps of the HIV-1 and SIV life cycles (even when pseudotyped with viral envelopes that have broad cellular tropism, such as the vesticular stomatitis virus glycoprotein (VSVg)) that cannot be fully explained by the differences in receptor-envelope binding [149, 153]. Indeed, the existence of cellular factor(s) within the intracellular milieu that restricts HIV and SIV infection at a post-entry, pre-integration step in non-permissive cells may provide a plausible explanation to reconcile these observations.

In mice, an example of such a host cell retrovirus-inhibiting factor is found in the case of the Friend virus susceptibility factor 1 (Fv1), a mouse gene product that can specifically block certain strains of the murine leukemia virus (MLV) [154, 155]. The Fv1 protein is derived from an ancient endogenous mouse retroviral gag gene, and disables incoming virions by binding to the MLV capsid (CA) immediately following entry into the cytoplasm, thereby preventing the virus from progressing through the remainder of the early steps in its replicative

cycle. This phenomenon, often referred to as the "Fv1 phenotype" is saturable, and the addition of high levels of incoming virions (infection at very high MOI) can overcome the resistance to infection in non-permissive cells [156, 157]. Interestingly, this Fv1 phenotype is also observed in certain human cells lines that are non-permissive for MLV infection and is caused by a genetically unrelated factor called Ref1 [158, 159].

In the Fv1 phenotype of MLV, a single-amino-acid residue in the CA protein of the incoming virion determines the N- or B-cell tropism of the virus. B-tropic MLV efficiently infects BALB/c cells but has only limited ability to replicate in NIH-3T3 cells, showing about a 100-fold lower titer. The opposite is true for N-tropic virus. The particular Fv1 locus in the host determines whether a cell will be of the B- or N-type. The protein product of Fv1 gene is presumed to interact with CA in the infecting virus, in a manner that is affected by this single amino acid change in the virion. While the Fv1 gene has recently been cloned [158], its precise mechanism of action remains to be elucidated. However, it is known that the MLV CA protein is a component of the viral preintegration complex (PIC) in newly infected cells [160], reinforcing the proposed mechanism of how the mutations in MLV CA can block downstream events in the viral life cycle such as the completion of reverse transcription and nuclear import. The importance of the retroviral CA protein to determining the viral tropism is

underscored by the observation of cyclophilin A binding to the CA of HIV-1 [161]. Treatment of virus-producing cells with cyclosporine, a drug that blocks cyclophilin A binding to CA and prevents its incorporation into budding virions, results in non-infectious virus that is blocked at an early step of the viral life cycle [162, 163]. Cyclophilin A binds to a loop in CA containing proline 90 in an unusual trans-conformation [162]. How this binding affects virion infectivity is unknown, but there may be a causal relationship between cyclophilin A binding to CA and the activity of a soluble factor that blocks lentivirus infection. Others suggest that the biological function of cyclophilin A binding to CA might be to destabilize the shell of CA proteins, thereby facilitating core disassembly during uncoating and allowing the infecting virion to proceed smoothly through the remainder of its replication cycle [164].

Might there be a host cell factor that demonstrates a similar virus-restricting phenotype in target cells for simian and human immunodeficiency viruses? Our recent studies have indicated that there does exist in certain primate cells a saturable inhibitor of primate lentiviruses, which we have termed Lv1 (Lentivirus susceptibility factor 1), that blocks HIV and SIV replication at an early step of the virus life cycle in non-permissive cells [149]. Recently, screening of a rhesus monkey cDNA library has identified tripartite interaction motif 5 -alpha (TRIM5-α) as a factor that confers resistance to HIV-1 infection [165].

Subsequent experiments have revealed that Lv1, Ref1, and TRIM5-α are most likely the same molecule, as expression of TRIM5-α confers resistance to lentivirus infection to otherwise permissive cell types [166]. The work presented in this thesis provided the first proof that Lv1/ TRIM5-α restriction occurs soon after the virus enters the infected cell, during the earliest steps of reverse transcription.

II. Lv-1/TRIM5-α restriction occurs at the step of early reverse transcription

A schematic diagram of the real time PCR assays that were developed to measure the various viral DNA intermediates (early and late reverse transcripts and 2-LTR circles) is shown in Figure 10. A more detailed description of these assays, including the locations of PCR primers and molecular beacons can be found in Chapter 6 Materials and Methods. In order to reduce the possibility of that contaminant DNA from the media used to make the viral stocks (i.e. leftover DNA from HEK 293T cell transfections) might confound our measurements of the viral DNA intermediates (specifically reverse transcription products) from infected cells, we developed a sensitive real time PCR assay to monitor the presence of this DNA species in our samples [Figure 10].

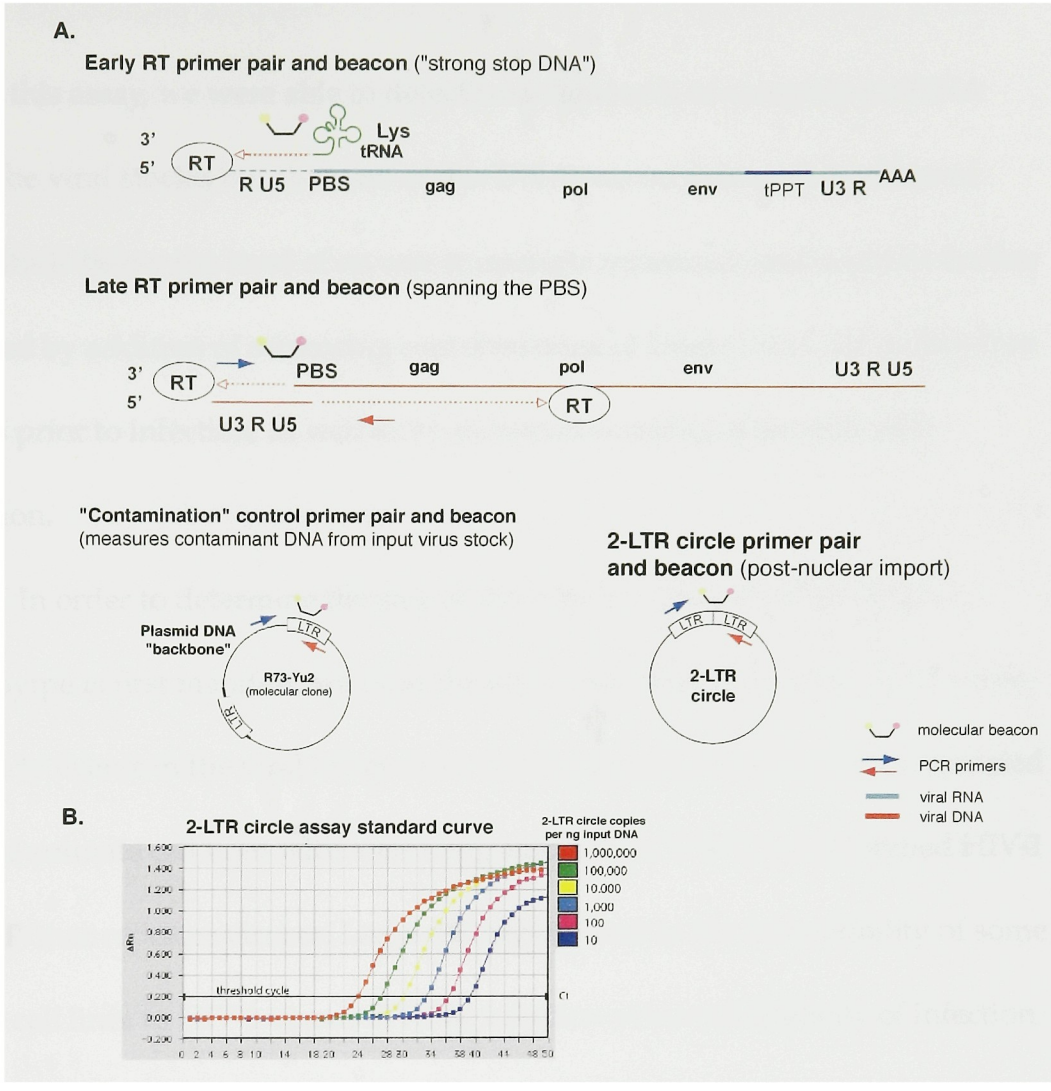


Figure 10. Description of Real Time PCR assays. Schematic diagrams depict the real time PCR assays used to measure various viral DNA intermediates during HIV infection. **A)** The Early RT primer set amplifies a small stretch of viral DNA preceding the primer binding site (PBS) and measures "strong stop" reverse transcripts that occur before the first template strand switch. The Late RT assay uses a reverse primer that anneals downstream of the PBS, giving an estimate of the full length reverse transcribed viral DNA. The 2-LTR circle assays utilizes a primer set and beacon that spans the LTR "junction" following the end-joining ligation reaction that occurs once the viral DNA is in the nucleus. A "contamination" control PCR assay with one primer specific for the plasmid DNA "backbone" of R73 and other specific for a region in the 5'LTR allows the accurate quantification of the amount of viral DNA carried over from the transfection used to make the viral stocks. **B)** An example of a standard curve generated using a 2-LTR circle plasmid as the template in this assay, starting with one million copies per nanogram of input DNA and 10-fold serially diluted to 10 copies per nanogram.

Using this assay, we were able to detect small amounts of contaminant DNA from the viral stocks, but the level of this DNA was on average 2-3 orders of magnitude below the level of reverse transcripts measured, and could be further reduced by addition of increasing concentrations of Dnase I enzyme to the virus stocks prior to infection, as well as by increased washing of the cells after infection.

In order to determine the step of the viral life cycle at which the Lv1 phenoytpe is first manifest, we used the above real time PCR assays to monitor the early events in the viral life cycle in various primate cells. We first inoculated several primate cell lines with increasing amounts of VSV-G-pseudotyped HIV-1 and SIVmac reporter viruses. Large differences exist in the susceptibility of some of the cell lines to HIV-1 versus SIVmac infection at low multiplicity of infection (MOI= 1). Specifically, whereas the human cell line HeLa was approximately equivalently infected by HIV/GFP and SIV/GFP, Rhesus monkey (Rh.F) and Owl monkey (OMK) cells were infected at an almost 100-fold greater frequency by SIV/GFP than by HIV/GFP. In contrast, the squirrel monkey cell line, Pindak, exhibited precisely the opposite phenotype. The African green monkey (CV-1) cell line was almost equivalently infected by each of the viruses, although both appeared less infectious on CV-1 cells at low MOI as compared with HeLa cells.

Accordingly, we then infected HeLa, OMK, and Rh.F cells with various doses of HIV GFP beginning with an MOI of 1000 with four 10-fold serial dilutions dilution until an MOI of 0.1 and then measured the accumulation of viral DNA intermediates at each dose. Analogous to the result seen with GFP expression measured by fluorescence activated cell sorting (FACS) analysis, OMK and Rh.F cells produced dramatically less reverse transcribed DNA following HIV GFP infection when compared to permissive HeLa cells [Figure 11a]. The dramatic species-specific restriction conferred by Lv1/TRIM5-α is even more clearly evident when one looks at the step of late reverse transcription [Figure 11b] or at the viral 2-LTR circles which form following nuclear import (not shown). Indeed, 2-LTR circles were only detected in the permissive HeLa cells (72 hours post-infection), and no circles were evident from the non-permissive Rh.F and OMK cell infections. This reinforces the assertion that Lv1/TRIM5-α restriction truly occurs at a step that is prior to nuclear entry, and may point to a temporal association between reverse transcription and nuclear import. For example, Lv1/TRIM5-α binding to capsids would not only prevent the conformational changes necessary to form active reverse transcription complexes (and thus block reverse transcription), but would also prevent the formation of pre-integration complexes competent for nuclear import. While the absence of 2-LTR circle intermediates in the non-permissive HIV infected cells

suggests a defect in nuclear import accompanies the block to reverse transcription, future studies that seek to directly visualize Lv1/TRIM5-α bound HIV cores will be needed to confirm this assumption.

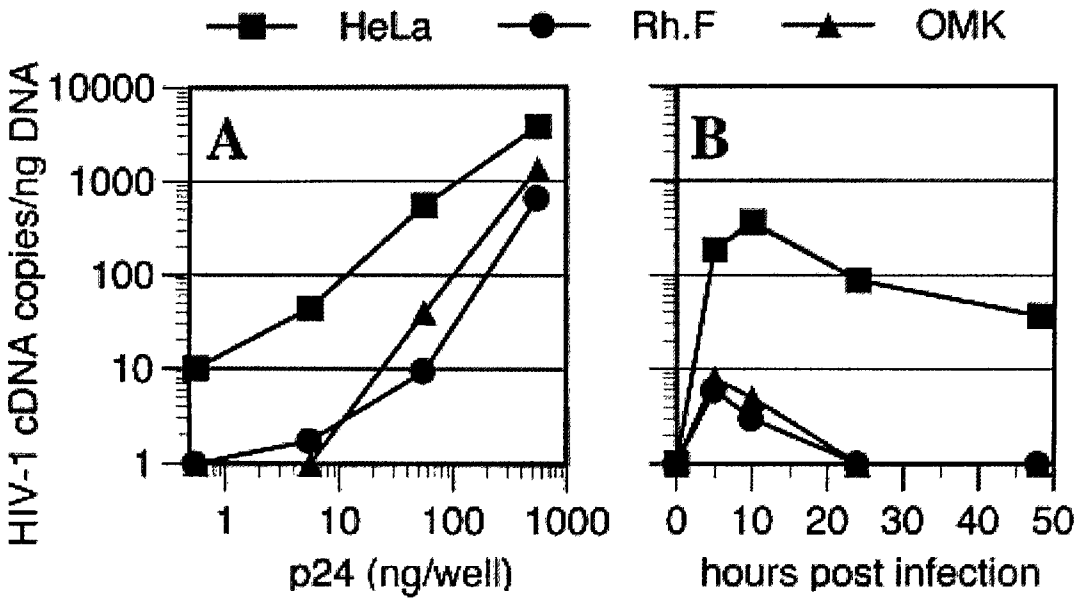


Figure 11a. Titration of VSV-HIV GFP on non-permissive cells reveals Lv1/TRIM5-alpha restriction is blocked at the step of early and late RT. Reduced accumulation of HIV-1 cDNA in nonpermissive primate cells is evident at low MOI. **A)** HeLa, Rh.F, and OMK cells were inoculated with varying amounts of VSV-G-pseudotyped HIVGFP and total DNA was isolated and reverse transcripts measured by real-time PCR 48 h later. Alternatively, **(B)** cells were inoculated with 25 ng (MOI ~0.1) of HIV-GFP, and reverse transcripts were measured at the indicated times after infection [62]. Data are representative of three independent experiments.

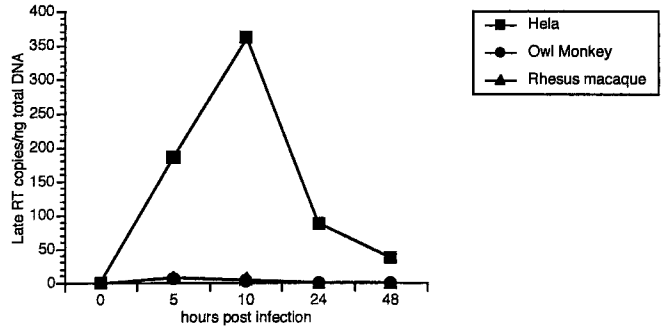


Figure 11b. Late reverse transcription is impaired in non-permissive primate cells. HeLa, OMK, and Rh.F cells were inoculated with 25 ng of HIV-GFP as above and total DNA was isolated 48 hours after infection and subjected to real time PCR analysis of completed late reverse transcripts. The inhibitory effect of Lv1/TRIM5-alpha produces a dramatic reduction in late reverse transcription in non-permissive OMK and Rh.F cells, which coincides with a lack of viral gene expression (GFP) as measured by FACS analysis, and a reduction in 2LTR circle copies. Data are representative of three independent experiments.

CHAPTER 3. NUCLEAR TRANSLOCATION OF HIV-1

Defining the role of HIV-1 integrase during viral nuclear import

Integrase has been mutagenized extensively in the context of numerous functional studies, but a systematic set of mutants has not yet been employed to localize determinants regulating its nuclear import. In previous studies [167] the integrase coding region was fused to the carboxyl-terminus of enhanced green fluorescent protein (EGFP), and the construct was transfected into HEK 293T cells. As expected, the 60 kD chimeric protein (EGFP-IN) localized predominantly to the nucleus in regions distinct from the nucleolus. An extensive set of charged-cluster-to-alanine mutations and carboxyl-terminal deletions were then introduced into the integrase portion of the EGFP-IN construct, and their effects on its sub-cellular localization determined. Alanine substitutions within the carboxyl-terminus of IN spanning residues 236-40 and 262-266, resulted in complete exclusion of EGFP-IN from the nucleus [167].

Mutagenesis of native integrase or transfer of the integrase carboxyl-terminal domain to otherwise cytoplasmic proteins [shown in Figure 12] confirms the existence of a potent NLS in this region. Remarkably, the charged residues do not by themselves confer NLS activity, as appending small 10-20 mer peptides corresponding to these regions within IN were not able to redirect

excluded eGFP-MBP fusion proteins to the nucleus [167]. Therefore it appears that the NLS activity of IN requires the structural integrity of a three dimensional SH3-like fold previously determined by others to exist in the carboxyl-terminal domain of integrase [168]. In addition, it is important to note that alignment of the amino acid sequences from over five hundred different primary isolates of HIV, spanning several of the known viral quasispecies including clades A, B, C, D, E, and G shows that this region of integrase is highly conserved, especially at the amino acid residues that we have identified as being critical for NLS activity [Figure 13]. This may underscore the importance of this region to the viral life cycle, specifically the residues critical to NLS function, as all viral subtypes would need to conserve this IN NLS region in order to interact with common cellular nuclear import machinery allowing the PIC to make it to the nucleus to become stably integrated into host chromosomal DNA. Indeed, the evoluntionary importance of this structure to the virus could be significant, as the residues that define the NLS element are highly conserved in multiple primate and non-primate lentiviridae. The conservation is complete in placement and sequence at the specified positions within the integrase protein including residues K236, R262, R263, K266, and partially at position K240.

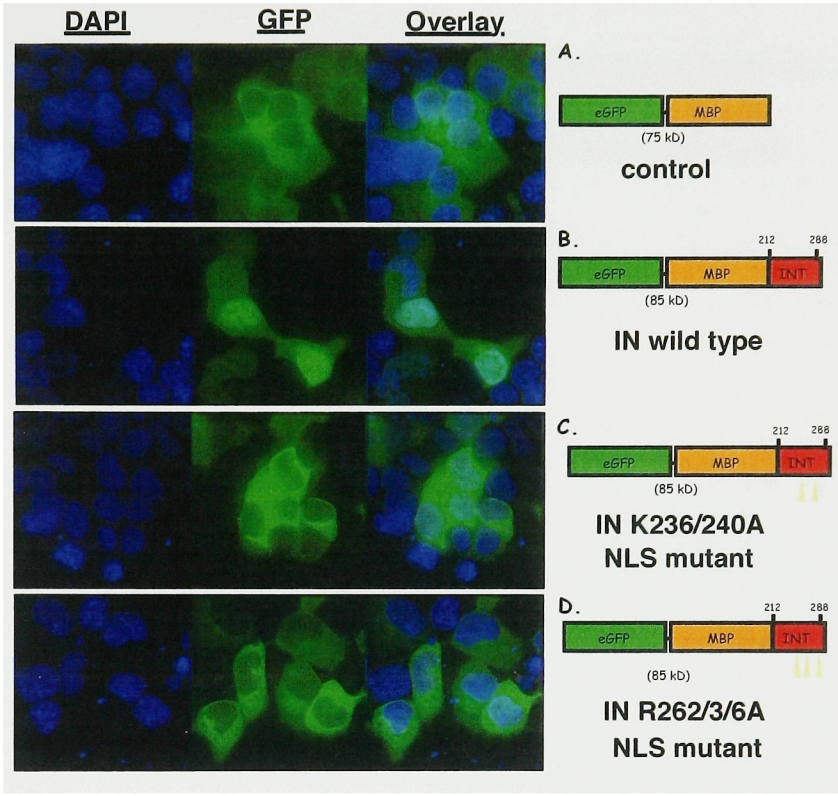


Figure 12. The C-terminal domain of HIV-1 integrase encodes an NLS activity that localizes large, otherwise cytoplasmic proteins to the nucleus. HEK 293T cells were transfected with DNA constructs encoding a fusion protein of enhanced green fluorescent protein (eGFP) and maltose binding protein (MBP) of 75 kiloDaltons, in excess of the nuclear pore exclusion limit (50-60 kD). The addition of the last 76 amino acids of HIV-1 integrase (the entire C-terminal domain) to this eGFP-MBP fusion resulted in the nuclear localization of this otherwise cytoplasmic protein. Inclusion of a double (K236/240A) or triple (R262/3/6) mutation in the appended IN C-terminus resulted in nuclear exclusion. **A)** control eGFP-MBP alone, **(B)** eGFP-MBP-IN-WT, **(C)** eGFP-MBP-IN-K236/240A, **(D)** eGFP-MBP-IN-R262/3/6A. All cells are shown at 63X magnification and each panel is representative of 50-100 eGFP-positive cells per field, four fields per slide, and representative of three separate experiments.

For the non-primate lentiviridae EIAV, FIV, BIV and Visna virus, this overall pattern of basic residue conservation within the integrase C-terminus is noticeable as well. Generally, clusters of basic residues are roughly 25-30 amino acids apart with hydrophobic residues involved in maintaining both the SH3 monomeric structure and the SH3:SH3 dimer interface notably preserved.

Frequency of Substitution (IN C-terminus, residues 220-270)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
I	Q	N	F	R	V	Y	Y	R	D	S	R	N	P	L	W	K	G	P
18	8	35	1	11	1	2	20	3	1	48	4	35	3	*	1	3	1	1

20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
A	K	L	L	W	K	G	E	G	A	V	V	I	Q	D	N	S	D	I
0	11	2	3	0	1	0	1	1	0	2	2	8	1	4	17	40	*	3

39	40	41	42	43	44	45	46	47	48	49	50	51
K	V	V	P	R	R	K	A	K	I	I	R	D
2	6	9	0	1	2	4	*	1	1	8	85	10

* >100 substitutions

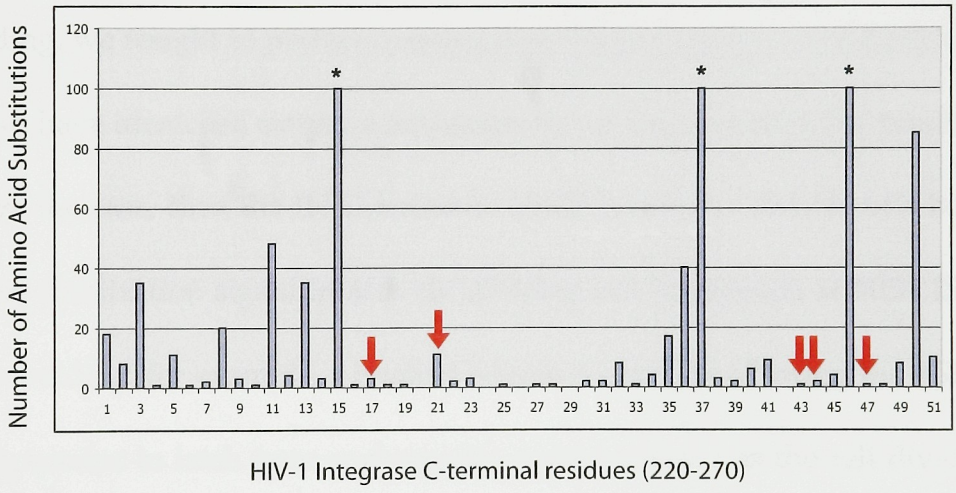


Figure 13. Conservation of Amino Acid Sequence in the C-terminal domain of HIV-1 Integrase. Five hundred and twenty HIV isolates across clades A, B, C, D, E and G were aligned in the pol gene region corresponding to the last 50 amino acids of the integrase protein. Overall, with the exception of 3 residues that displayed great variation (indicated by an *), there is a high degree of sequence conservation in this region, especially at the residues that we have identified as relevant to viral nuclear import (red arrows).

I. The carboxyl terminus of HIV-1 integrase specifies a transferable nuclear localization signal (IN NLS) that functions in primary cells

As mentioned previously, introducing mutations in the integrase region of *pol* can often have pleiotropic effects on essential viral activities other than nuclear import, such as PIC assembly or reverse transcription. As the integrase SH3 domain has been shown to bind DNA non-specifically *in vitro* [169-172], and one of the set of mutations that we have identified as critical for NLS activity overlaps a residue (Lys at position 264) that is important for this non-specific DNA binding, we sought to perform experiments to preclude the possibility that the NLS we have identified simply coordinates host chromosomal DNA binding. If this were the case, then the IN C-terminus could mimic the activity of a bona fide nuclear localization signal in actively dividing cell lines (such as HEK 293T cells) by providing, for example, a nuclear retention function allowing the eGFP-IN fusion proteins to latch onto euchromatin nonspecifically as the cell divides. Figure 14 shows that the integrase NLS element is capable of providing nuclear import activity to otherwise cytoplasmic eGFP-MBP fusion proteins even in non-dividing cells of primary origin (immature dendritic cells and CD4+ T cells); evidence that the integrase NLS is operational for import when the nuclear membrane remains intact and when no transient access to chromosomal DNA during cell division is available. The process of nucleofection that was used to

introduce the eGFP-MBP-IN plasmid DNAs into non-dividing DC and T cells produces transient porosity of the cell's plasma membrane but the nuclear membrane remains intact [173], and, while considerable cell death is evident in the samples following this procedure, there is no evidence of cell division as measured by CSFE staining and FACS anaylsis of samples after nucleofection (not shown). Therefore, its is unlikely that the apparent karyophilic activity provided by the HIV IN C-terminus is simply an artifact of experiments performed in dividing cells, which highlights the potential importance of this domain of integrase as a facilitator of viral nuclear import during natural infection *in vivo*.

II. Kinetics of HIV IN NLS mutant viruses in dividing and non-dividing cells

Reconstruction of these integrase NLS mutants into an otherwise infectious HIV-1 proviral clone (R7-3 with a macrophage tropic (Yu2) or T cell tropic (HXB-2) envelope) resulted in a replication-defective phenotype in all cell types tested. Figure 15 shows the results of infection or the T lymphoblastoid cell line CEM-SS cells, primary human monocyte-derived macrophages (MDM), and primary CD8-depleted PHA-activated peripheral blood lymphocytes (PBLs) with either macrophage tropic or T cell tropic HIV variants containing mutations to

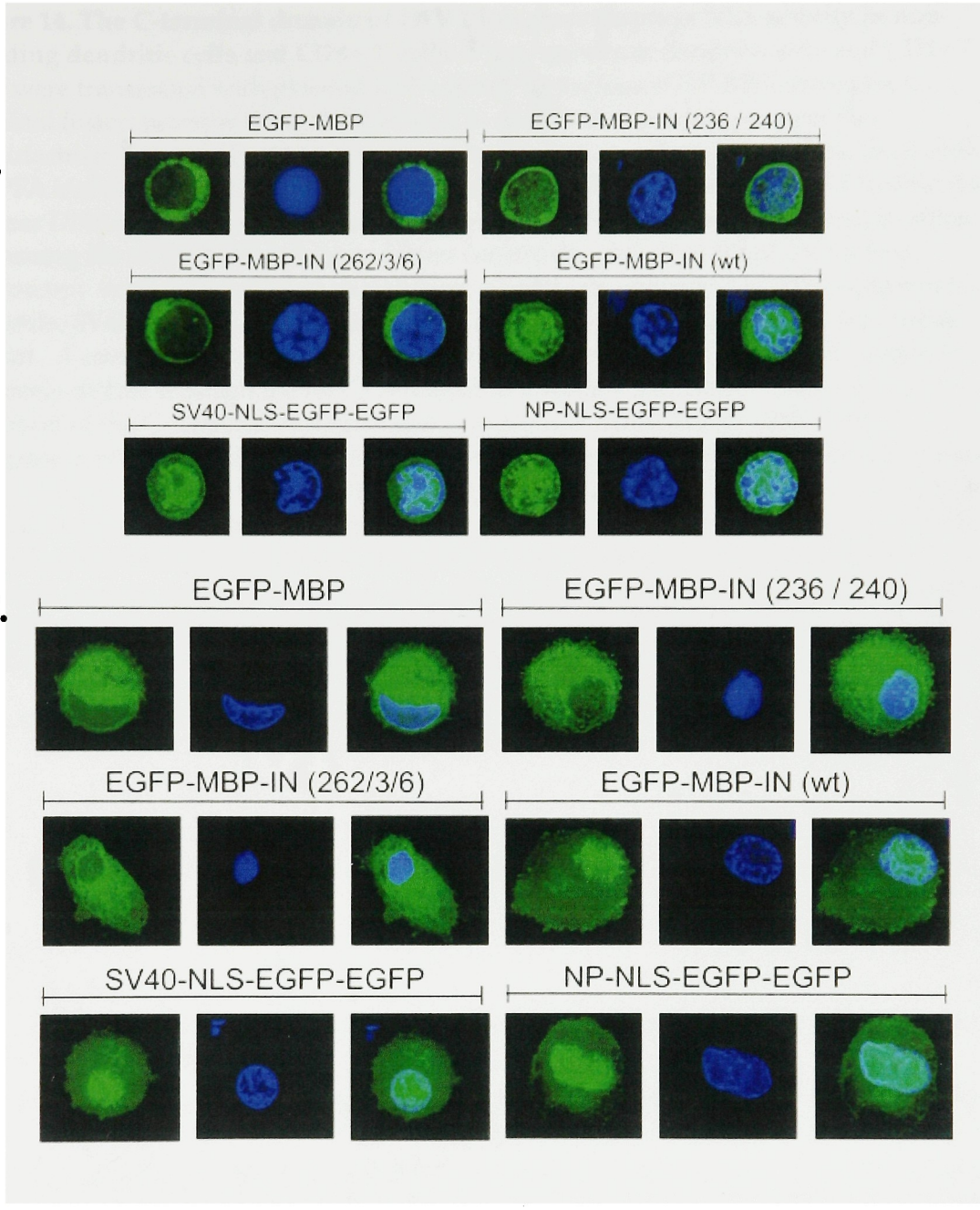


Figure 14. (Caption continued on page 67).

Figure 14. The C-terminal domain of HIV-1 integrase displays NLS activity in non-dividing dendritic cells and CD4+ T cells. Primary human dendritic cells and CD4+ T cells were transfected with plasmid DNA encoding various eGFP-MBP-Integrase C-terminal fusion proteins using the AMAXA Nucleofection procedure as per the manufacturer's protocol. At 12 hours post-nucleofection, cells were washed, fixed with 4% PFA and mounted onto microscope slides with medium containing DAPI to stain the nuclear DNA (blue). Cells that were positive for eGFP expression (green), and therefore expressing the eGFP-MBP-Integrase fusion constructs, were visualized by confocal microscopy using a DeltaVision microscope. Constructs specifying eGFP conjugated to either the SV40 virus NLS or nucleoplasmin NLS were included as controls for nuclear import. A construct encoding the eGFP-MBP fusion protein alone (75 kD, too larger to passively diffuse through the NPC) was used as a control for nuclear exclusion. **A)** Addition of the C-terminal 76 amino acid polypeptide (residues 212-288) of HIV-1 integrase confers an NLS activity to an otherwise cytoplasmic protein in primary human CD4+ T cells. **B)** The C-terminal HIV-1 Integrase NLS is also functional in non-dividing primary human dendritic cells. Mutation of the residues previously shown to affect HIV -1 nuclear import (K236/240A and R262/3/6A) in the context of this appended C-terminal domain resulted in nuclear exclusion of the fusion protein. All cells are shown at 100X magnification and each panel is representative of 50-100 GFP positive cells per field, four fields per slide, and a minimum of three separate experiments.

the defined NLS regions K236/240A and R262/3/6A. Examining the infected cell supernatants for the presence of the viral p24 Gag antigen indicates that these mutations result in a non-productive infection when compared to cognate wild type viruses. Similarly, transfer of infected cell supernatants taken from each cell type after seven days in culture produced the same pattern of infection when added to fresh cultures of target cells that are permissive for HIV infection such as PHA-stimulated CD8-depleted PBLs. Thus, incorporation of this double (K236/240A) and triple (R262/3/6A) mutation into an otherwise infectious proviral molecular clone results in a virus that is replication-defective, presumably because viral nuclear import is unable to proceed to completion.

III. Real time PCR analysis of viral DNA intermediates after infection with IN NLS mutant viruses

In order to examine the effects of NLS mutants on specific early stages of the HIV life cycle, we developed a series of sensitive, stage-specific real-time PCR-based assays that quantitatively measure progression through reverse transcription and nuclear import. In agreement with other kinetic PCR assays [174], calibration of a typical spreading infection with wild-type virus exhibits a 2- to 4- log increase over baseline for each of the viral DNA intermediates over 48 hours, as the stage-specific products appear sequentially.

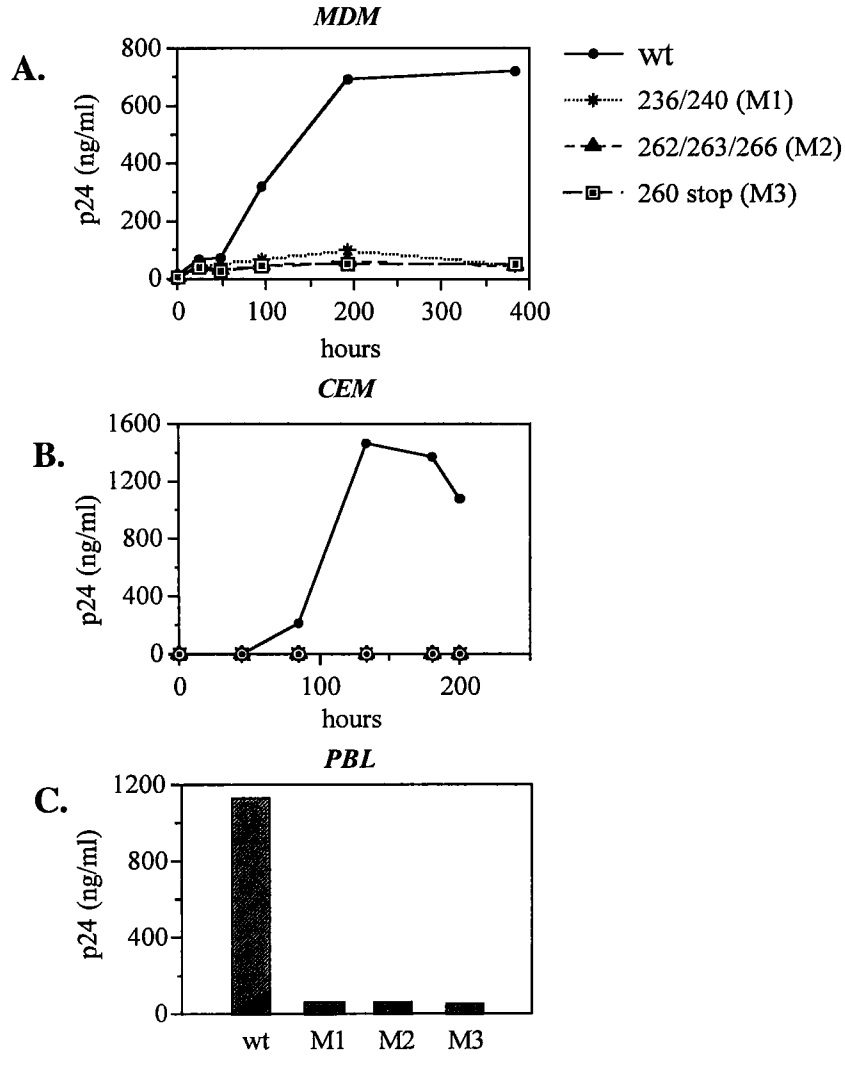


Figure 15. Replication kinetics of HIV IN NLS mutant viruses in dividing and non-dividing cells. Non-dividing primary human monocyte-derived macrophages (MDM) and CD8-depleted peripheral lymphocytes (PBL) were infected with HIV molecular clones derived from the HXB2 strain (R7-3), containing our NLS mutations in the integrase region of the *pol* gene, and pseudotyped with either a T cell-tropic or M-tropic (Yu2) HIV envelope. After 6 hour initial incubation with virus, cells were washed and media was collected at the indicated time points **A)** MDM infected with wild type (WT) virus produced p24 consistent with a productive spreading infection while NLS mutant viruses did not (K236/240A=M1, R262/3/6A=M2, 260stop=M3). **B)** The NLS mutant viruses are replication defective in the CEM-SS lymphoblastoid cell line. **C)** CD8-depleted PBL do not support replication of the NLS mutant viruses, while WT virus readily replicates in these cells (media assayed at a single time point 8 days post infection in panel C). Data are representative of five separate experiments.

Notably, the copy number per cell equivalent of each stage-specific product is approximately 1-2 orders of magnitude less than the previous step, with early reverse transcripts the most abundant and 2-LTR circles the least, highlighting the relative inefficiency of the conversion of viral RNA into nuclear viral DNA species.

Single-cycle infections with wild type and NLS-mutant R5 and X4 viruses were performed in CD8-depleted, activated primary lymphocytes and MDM, respectively. In all cases, the integrase strand-transfer inhibitor, L-731,988 [175], was added to the infections. This inhibitor has no effect on the nuclear localization of the enzyme or viral DNA accumulation in the nucleus but renders it catalytically inactive at the strand-transfer step [175]. Inclusion of the strand transfer inhibitor allowed a comparison of all viruses during a single round of infection. In addition, it increased the utility and accuracy of the 2-LTR circle assay as a marker for PIC nuclear import by eliminating potential variations in integration efficiency between the wild type and mutant viruses. This is important for avoiding confounding effects, since the frequency of viral integration is inversely correlated with the accumulation of the circular viral DNA species found in the nucleus [42, 175].

Figure 16 shows that while wild type and integrase NLS-mutant viruses are able to complete the late stage of reverse transcription with equal efficiency,

all NLS-mutant viruses exhibit a severe defect in proviral nuclear import, as measured by 2-LTR circle formation in primary human MDM. The same pattern was observed in CD8-depleted PBL [Figure 17].

In order to ensure that the presence of a pharmacological integrase inhibitor did not confound our results by exerting possible pleiotropic effects, experiments were repeated in the absence of the drug. Here, MDM were infected with wild-type and NLS-mutant viruses containing an additional mutation, IN D116A, which by itself has no effect on nuclear localization of the integrase protein, reverse transcription, or transport of the viral genome into the nuclear compartment, but prevents integration and further progression through the viral life cycle [42]. Results using these viruses were the same as those obtained using the small molecule integrase inhibitor, with a specific and severe two-log defect evident at the level of 2-LTR circle formation. Thus, the functional integrase NLS described here is necessary and sufficient for efficient nuclear accumulation of the viral genome in cell types reflective of the natural target cells for HIV infection *in vivo*.

To rule out any effects the amount of virus used to initiate an infection might have on the interpretation of our results, we determined the level of late reverse transcripts over a two-log range of X4 and R5 viral input concentrations (1-243 ng p24).

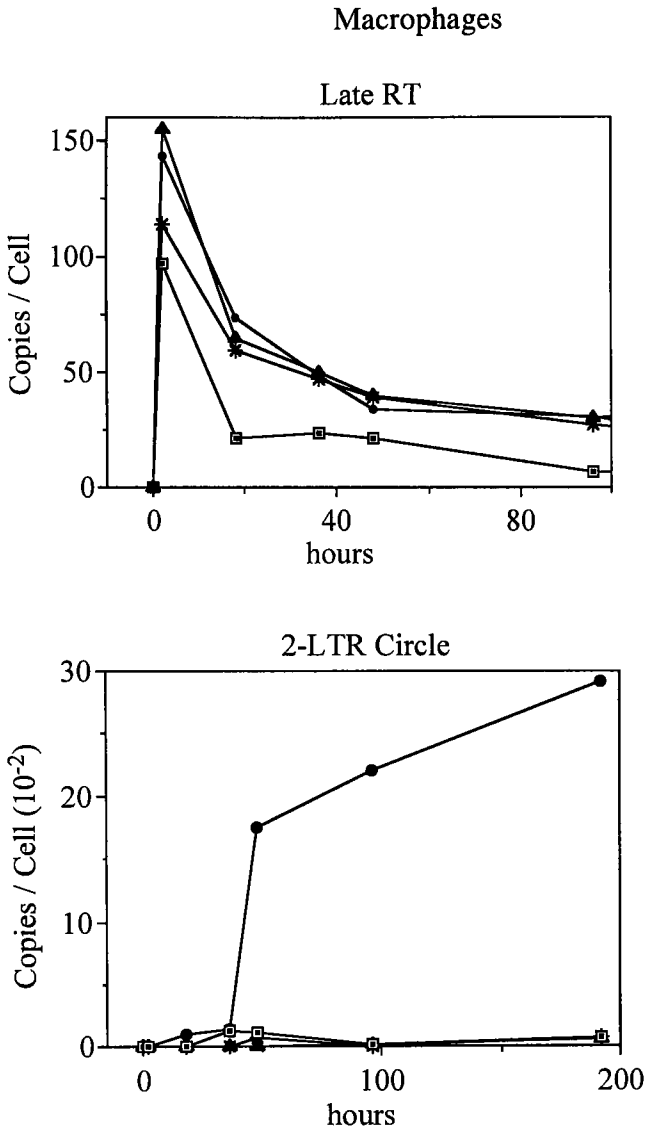


Figure 16. Real time PCR analysis of viral DNA intermediates in primary human macrophages following infection with HIV IN NLS viruses. Primary human macrophages were infected with macrophage-tropic HIV viruses containing the previously identified integrase NLS mutations K236/240A, R262/3/6A, and 260stop in the presence of the diketo integrase inhibitor L-731,988. After addition of viral stock, cells were washed with phosphate buffered saline, then fresh media was added and the infected macrophages were kept in culture with drug for total of 8 days. Total genomic DNA was isolated at 0, 6, 24, 48, 96, and 196 hours post infection and viral DNA intermediates for late reverse transcription **(A)** and 2-LTR circles **(B)** were quantified via real time PCR. With the exception of the 260stop truncation mutant, the IN NLS mutations displayed little or no defect in reverse transcription, while there was a marked decrease in 2-LTR circles compared to wild type virus (solid black circles), suggesting that these viruses are defective for nuclear import. Results are representative of at least 3 separate experiments.

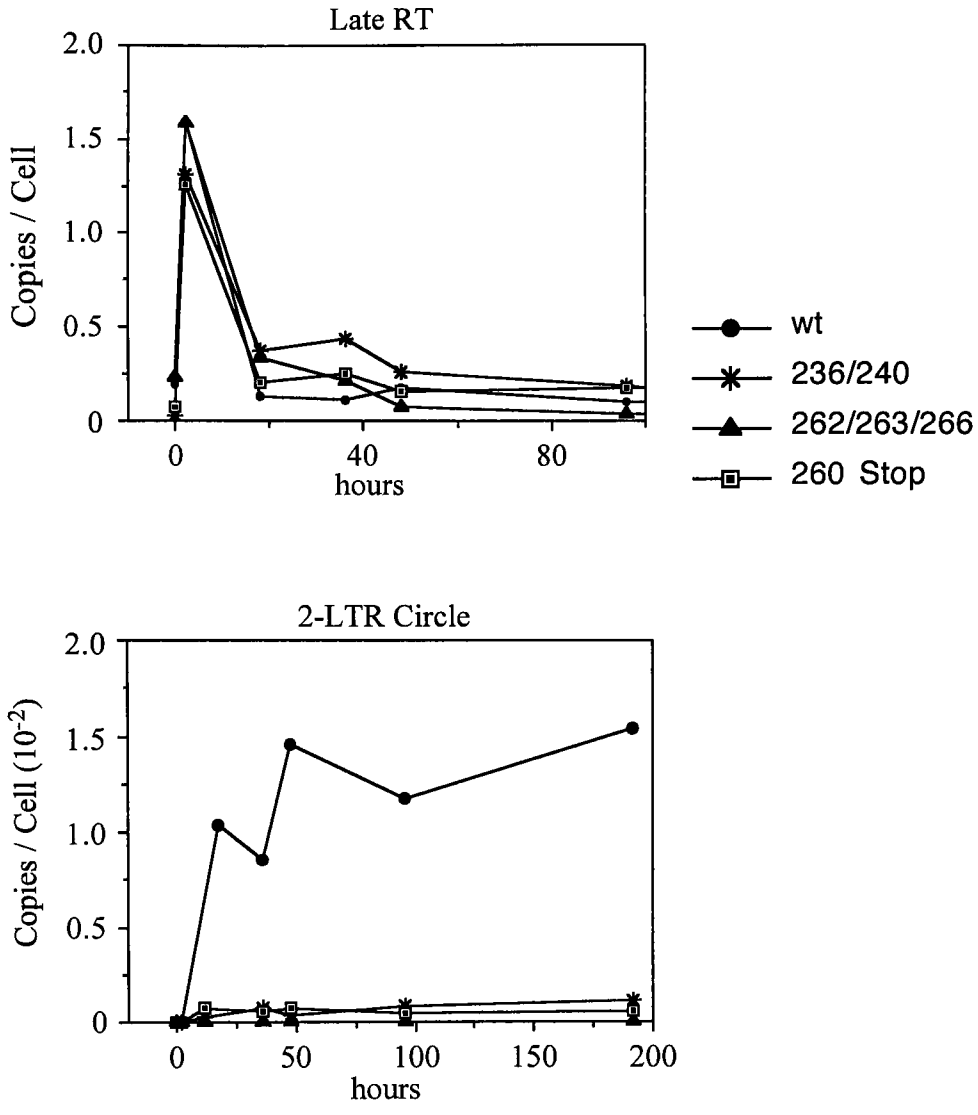


Figure 17. Real time PCR analysis of viral DNA intermediates in primary human CD8-depleted PBL following infection with HIV IN NLS viruses. CD8-depleted, PHA-activated primary human peripheral blood lymphocytes (PBL) were infected with T cell-tropic HIV viruses containing the previously identified integrase NLS mutations K236/240A, R262/3/6A, and 260stop in the presence of the diketo integrase inhibitor L -731,988. After addition of viral stock, cells were washed with phosphate buffered saline, then fresh media was added and the infected PBLs were kept in culture with drug for total of 8 days. Total genomic DNA was isolated at 0, 6, 24, 48, 96, and 196 hours post infection and viral DNA intermediates for late reverse transcription **(A)** and 2-LTR circles **(B)** were quantified via real time PCR. With the exception of the 260stop truncation mutant, the IN NLS mutations displayed little or no defect in reverse transcription, while there was a marked decrease in 2-LTR circles compared to wild type virus (solid black circles), suggesting that these viruses are defective for nuclear import. Results are representative of three separate experiments.

For example, viral cDNA is unstable in infected cells, with incompletely synthesized genomes preferentially targeted for degradation [176]. Thus, total cellular DNA was isolated six hours after infection and the amount of late reverse transcripts produced was determined for cultures of CD8-depleted PBLs and MDM. The accumulation of late reverse transcription products in the integrase NLS mutant is identical to that of the wild-type virus over the range of doses tested (data not shown), suggesting that any potential dose-dependent effects of viral innoculum on our reverse transcription measurements are minimal, and would not confound the measurements of 2-LTR circle products that formed downstream of the completion of reverse transcription.

Finally, to ensure that the role of integrase in viral nuclear import was independent of upstream events of the viral life cycle, we attempted to pharmacologically isolate and continuously monitor a single population of reverse transcripts for nuclear import. MDM were infected with either wild type or IN NLS mutant virus in the presence of the integrase strand transfer inhibitor L-731,988 as described above. Then, at 3 hours post-infection, the non-nucleoside reverse transcriptase inhibitor, UC-781 [177], was added to block further DNA synthesis, and the levels of reverse transcript and 2-LTR circle formation was monitored at various times post-addition by real-time PCR. Under these

conditions, both the production and stability of viral cDNA transcripts were identical in the wild type and mutant viral infections [Figure 18a]. However, 2-LTR circle formation (presumably from the nuclear import-competent PIC that formed prior to the addition of the RT inhibitor) was dramatically reduced for the IN K236A/240A virus relative to the wild-type, indicating a specific and severe defect for nuclear import when this stage of the viral life cycle is pharmacologically uncoupled from upstream and downstream events by treatment with the RT inhibitor UC-781 [Figure 18b].

IV. Preintegration complexes and purified proteins from IN NLS mutants are catalytically active in vitro

In order to prove that our integrase NLS mutants were competent to perform the normal obligate catalyses of the enzyme, purified versions of both wild type and NLS mutant codon-optimized integrase proteins (IN MOD) were made (courtesy of the lab of Dr. Daria Hazuda, Merck). These purified IN proteins were analyzed using the Quick IN assay for strand transfer described previously by Hazuda et al. [175, 178-181]. The K236/240A mutant was nearly equivalent to wild type protein in its ability to perform the strand transfer

reaction, while both the R262/3/6A and 260stop mutant were impaired (45-50% of wild type activity) in this assay [Figure 19b (top panel)].

In addition, we developed a quantitative real time PCR based assay for quantifying integration of the viral genome into a small (186 base pair) target DNA. Cytosolic extracts containing active pre-integration complexes were made from macrophages infected with the wild type and NLS mutants, incubated with a defined amount of the small target DNA, phenol:chloroform extracted and used as a template in our quantitative real time PCR assay for integration. Figure 19 describes the assay setup and shows the results of both sets of experiments with purified IN NLS mutant proteins or purified HIV PICs from the mutant NLS viruses. The K236/240A NLS mutant PICs showed no defect in their ability to integrate into target DNA compared to wild type PICs. However, a defect in integration activity was noticed for the R262/3/6A and 260 stop NLS mutants (50 and 75 percent, respectively) compared to wild type PICs. PIC from the D116A mutant, as well as PICs from cells infected with wild type virus and treated with 10 μM of the strand-transfer inhibitor L-731,988 were included as controls and produced background levels of integration as expected. Thus, the results of these experiments suggest that all of the NLS mutants are competent to some degree to catalyze the strand transfer reaction.

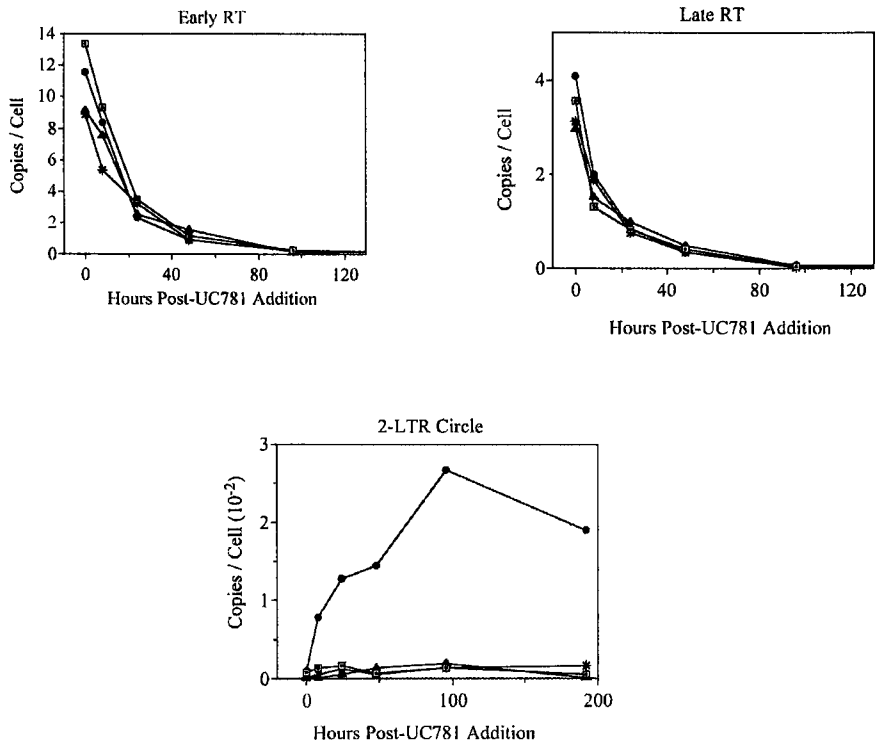


Figure 18. Pharmacological isolation of viral DNA intermediates at the step of reverse transcription reveals RT-independent effect of IN NLS mutants on nuclear import in primary human macrophages. Primary human macrophages were infected with M-tropic HIV containing the IN NLS mutations as well as the D116A active site mutation at a high MOI (10), then treated with a non-nucleoside reverse transcriptase inhibitor (UC -781) at 3 hours post-infection. Total genomic DNA was isolated at 0, 6, 12, 24, 48, 96, and 196 hours post-UC-781 addition and viral DNA intermediates for early and late reverse transcription and 2-LTR circles were measured by real time PCR. Under conditions where reverse transcription is inhibited, a marked decrease in 2-LTR circles is apparent for the NLS mutants compared to wild type virus, suggesting that these mutants are specifically impaired for the step of viral nuclear import. Data is representative of two separate experiments.

In the case of the mutants that were compromised for this catalytic activity (the R262/3/6A and 260stop mutants), this may be due to a decrease in their ability to bind the target DNA substrate, as residues within this region of IN have been implicated in DNA binding [169, 170]. As the K236/240A mutant was nearly equivalent to wild type integrase activity in both assays, its effects on nuclear import are independent of its ability to perform the enzymes' normal catalytic functions *in vitro*.

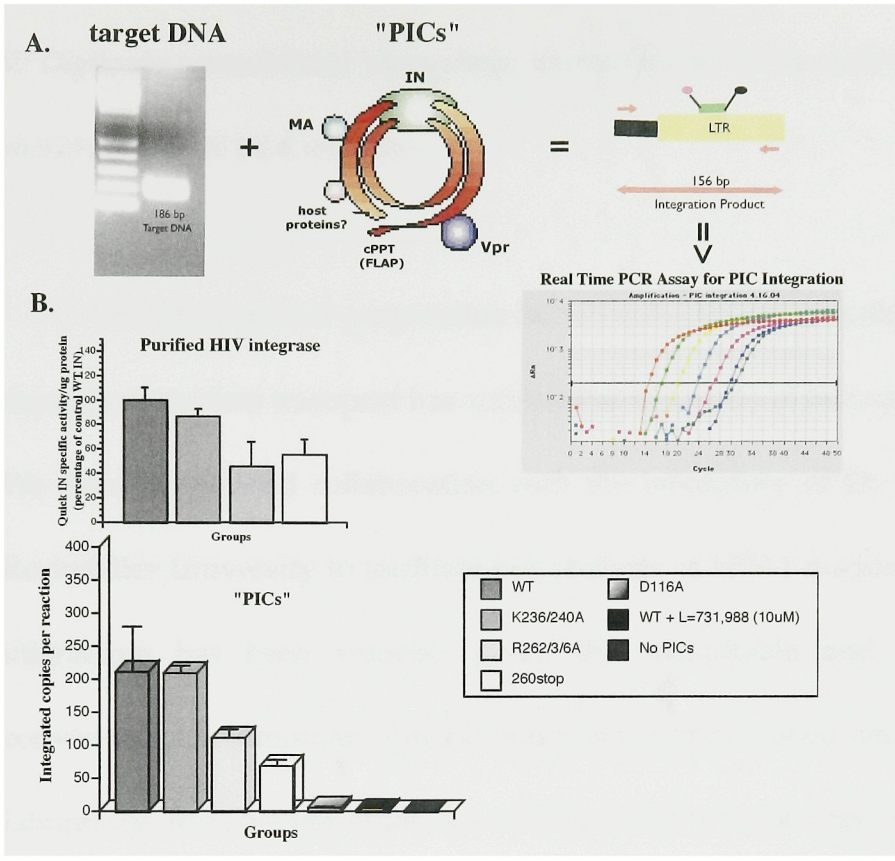


Figure 19. Pre-integration complexes from IN NLS mutant viruses are catalytically active *in vitro*. In order to test the ability of the NLS mutant PICs to perform other obligate catalytic functions of integrase, we added cytosolic extracts from cells infected with wild type or NLS mutant viruses to a small 186 base pair target DNA. **A)** A schematic diagram of the PIC integration real time PCR assay set up is shown. A forward primer annealing to a defined stretch of sequence in the target DNA was paired with a reverse primer and molecular beacon specific for the 5' LTR allowing for quantitative measurement of an approximately 156 bp integration product. An example of a standard curve generated with a plasmid containing this integration product is shown. **B)** The Quick IN integration assay of purified IN proteins was performed and data is expressed as percentage of wild type activity per microgram of protein. PICs from WT and NLS mutant viruses were incubated with target DNA for 2 hours at 37 degrees C, then integration products were phenol:chloroform extracted and quantified using the real time PCR assay described above. Both the R262/3/6A and 260stop mutants displayed decreased integration (50% and 30% of WT respectively), while the K236/240A mutant showed no integration defect. A D116A active site mutant, and wild type PICs treated with the integrase inhibitor L-731,988 were included as controls. Equivalent amounts of PICs (as measured by late RT products in the PIC preps) were added to 250 ng of target DNA in each reaction. The quick IN assay represents two independent experiments and the PIC integration experiment was repeated three times.

V. Digitonin-permeabilized macrophage nuclear import assay system reveals excluded phenotypes for IN NLS mutants

The cloning and preparation of recombinant factors required for signal-dependent nuclear transport has accelerated the study of intracellular trafficking. We have maintained collaboration with the laboratory of Dr. Gunter Blobel at Rockefeller University to facilitate our analysis of HIV-1 nuclear import and this interaction has been valuable given the remarkable and extensive set of conventional recombinant nuclear transport factors cloned and purified by this laboratory. To make use of this knowledge and the latest approaches to the study of intracellular trafficking, we have developed a semi-intact cellular based system centered on the selective soponification of the plasma membrane over that of the nuclear membrane. This system maintains the nucleus (and the nuclear membrane) structurally intact and in a state competent for signal-dependent nuclear pore translocation. Digitonin permeabilization, followed by extensive washing with isotonic transport buffer, literally "flushes" the soluble factors required for nuclear import out of the cell's cytoplasmic compartment. Specific factors required for nuclear transport of a given protein cargo or complex can be studied in detail by the addition of these factors back into the cell from

fractionated cytosol preparations or, depending on their availability, from a specific combination of the recombinant factors and transport factors of interest. In this reconstituted import system, proteins with active nuclear localization signals can be found in the nucleus within as early as thirty minutes after addition to permeabilized cells and reach more than a thirty-fold increase in concentration into this compartment [182].

In our experiments we used primary human macrophages as the target cells for permeabilization, as this cell type is relevant to HIV infection *in vivo*. The digitonin-permeabilized macrophage cell system reproduces authentic nuclear protein import in that it requires a functional nuclear localization sequence, ATP and cytosol, is temperature dependent, and is inhibited by wheat germ agglutinin (WGA), a molecule that binds the nuclear pore and is a potent inhibitor of nuclear transport *in vivo*. The results obtained using purified IN proteins [Figure 20] containing our NLS mutations, as well as PICs from cells infected with the NLS mutant viruses [Figure 21], have reinforced our belief that the carboxyl-terminal domain of integrase is indispensable for viral nuclear import, as the nuclear excluded phenotype we observed in the previously described studies of eGFP-MBP fusion proteins containing the NLS mutations can be recapitulated in this *ex vivo* system. Digitionin-permeabilized macrophages were incubated with nuclear transport mixtures containing

purified proteins or PICs (cytosolic extracts from infected macrophages) for 2 hours to allow nuclear import to occur, then cells were washed, fixed with paraformaldehyde (PFA), and immunohistochemically stained with monoclonal antibodies specific for the N-terminus of HIV integrase. Purified GST-GFP fusion proteins plus or minus the NLS peptide from nucleoplasmin (courtesy of Dr. Elias Coutavas, Rockefeller University) were subjected to the same conditions and visualized in cells in order to clearly define what a nuclear localized protein looks like inside of permeabilized macrophages. Other controls included

immunostaining with non-specific IgG antibody as well as uninfected cell cytosol containing no PICs. In addition, the nuclear pore blocking compound, wheat-germ agglutinin (WGA), was included in some of the nuclear transport assays, in order to show that PIC nuclear import is indeed conducted through the NPC [shown in Figure 21].

VI. Vpr-GFP tagged IN NLS mutant viruses display decreased nuclear localization

The labeling of HIV virions via incorporation of fluorescently-tagged Vpr molecules has previously been described [27, 183]. In order to directly visualized the sub-cellular localization of our NLS mutant virions during a live ongoing infection, viral stocks of Vpr-GFP labeled virions containing these mutations in

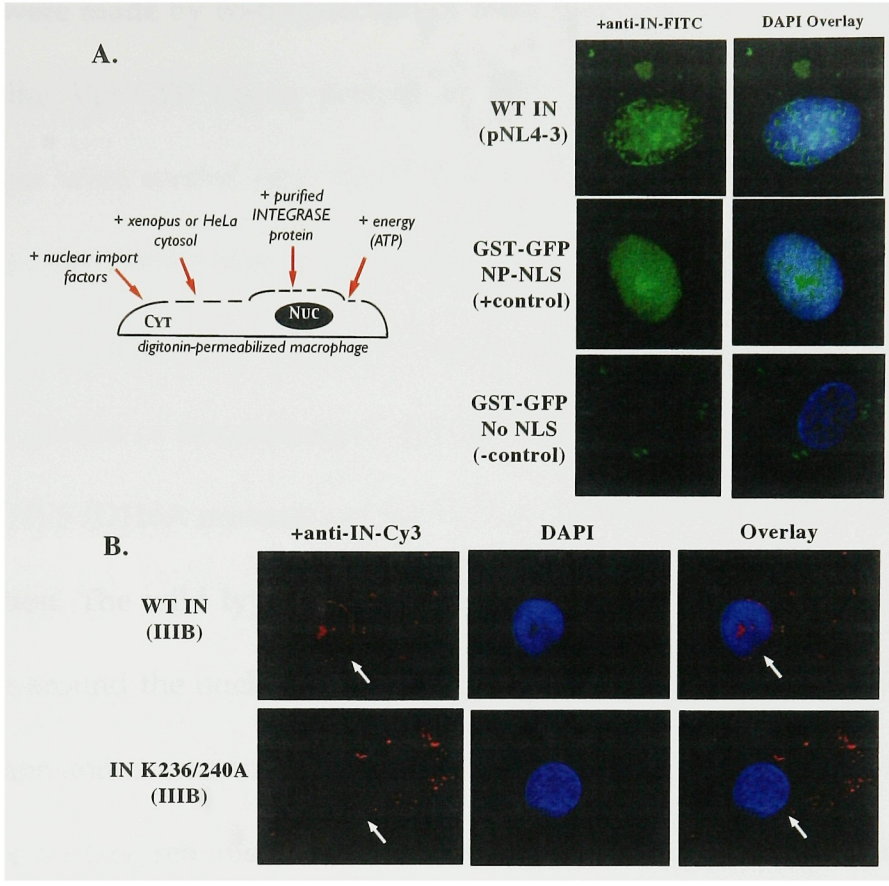


Figure 20. Digitonin-permeabilized macrophage nuclear import assay system reveals excluded phenotypes for IN NLS mutant purified proteins. Primary human macrophages were grown on poly-L-lysine coated 12mm round glass coverslips and permeabilized with digitonin (see Methods). Cells were washed with 1X transport buffer to remove the cytosol, then a mixture containing xenopus oocyte cytoplasmic extract, ATP energy source, RAN, GTP, and 1ug of purified HIV integrase was added and cells were incubated at 37 degrees Celsius to allow nuclear import to occur. **A)** A diagram of the permeabilized cell system, followed by results of initial experiments using purified WT IN from the pNL4-3 strain of HIV detected with an anti-IN monoclonal antibody (mAb6G5) and a tyramide- FITC (green) kit. A GST-GFP fusion protein +/- the nucleoplasmin (NP) NLS was included as a control for nuclear import. **B)** Wild type (WT) and K236/240A mutant purified IN proteins from the HIV IIIB strain were added to transport reactions on permeabilized macrophages, and stained with mAb6G5 followed by a tyramide-Cy3 detector. The K236/240A mutant displayed a nuclear exclusion phenotype when compared to WT (white arrows) similar to that seen in the eGFP-MBP fusion protein studies described previously. All cells were stained with DAPI to visualize the nuclear DNA (blue). Cells were visualized at 100X magnification on a DeltaVision microscope and each panel is representative of 30-50 permeabilized stained cells per field, four fields per slide, and three independent experiments.

IN were made by co-transfection of these plasmids along with a plasmid encoding the Vpr-GFP fusion protein in HEK 293T cells. Primary human macrophages were seeded onto special Petri dishes (containing a single glass microscope slide embedded in the middle), and GFP-tagged virions were continuously visualized by live-cell imaging over a 16 hour period. Figure 22 shows still photos of macrophages infected with macrophage-tropic (Yu2 env) wild type NLS (D116A mutant) and K236/240A NLS mutant viruses at 6 hours post infection. The wild type NLS viruses clearly demonstrate a propensity to congregate around the nucleus, a pattern which is evident as early as one hour post infection and characterized by circular configurations of GFP-labeled virions around the surface reminiscent of nuclear "rim staining" of nucleoporins like Nup358 (see Fig. 21). Meanwhile, the K236/240A mutant virions did not display the same pattern of nuclear localization in infected macrophages, with the majority of the particles appearing scattered throughout the cytosol. Close examination of time-lapse movies made from assembled images from the live-cell infections [Figure 30, attached DVD] shows that, while many virions appear to move towards the nucleus in the wild type NLS cultures, the Vpr-GFP molecules never actually enter the nucleus per se, and the particles seem to remain attached to the nucleus on the cyctoplasmic side.

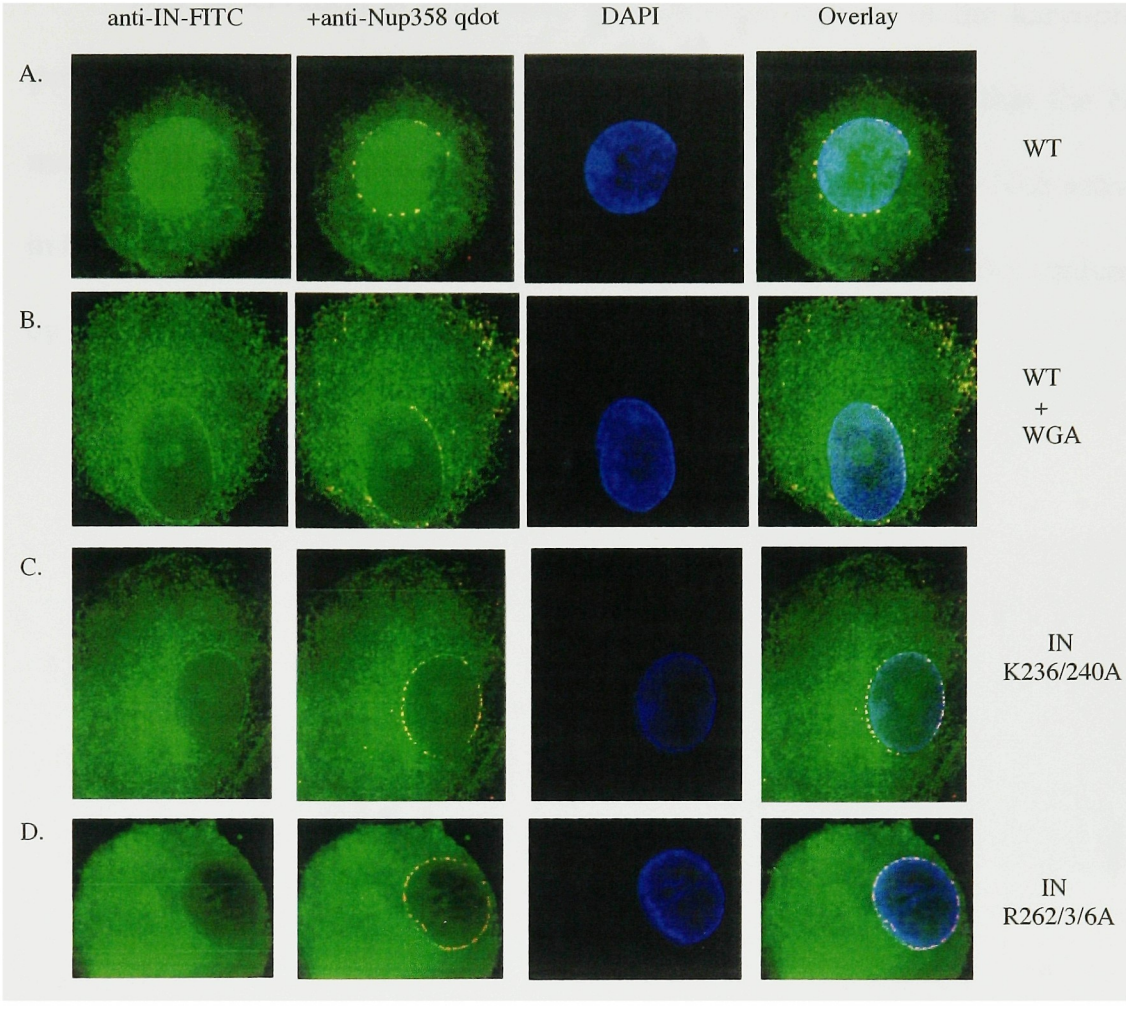


Figure 21. Digitonin-permeabilized macrophage nuclear import assay system reveals excluded phenotypes for IN NLS mutant pre-integration complexes. Three million macrophages were infected with a high MOI (100) of M-tropic HIV virus stock (wild type R73-Yu2 and IN K236/240A as well as R262/3/6A NLS mutants). Twelve hours following infection, a cytosolic extract was made from these infected cells and added to donor-matched digitonin-permeabilized human macrophages. Immuno-histochemical staining was performed using anti-integrase monoclonal antibodies (INT-2, INT-4, mAb6G5) with a FITC-tyramide detector (green). Nuclear pores were stained with anti-Nup358 polyclonal rabbit antibodies (C-288 from E. Coutavas) coupled to an anti-rabbit quantum dot 655 detector (red). Nuclei were stained with DAPI (blue) upon mounting. **A)** Wild type R73-Yu2 PICs. **B)** Wild type PICs + Wheat Germ Agglutinin (WGA), which blocks transport through nuclear pores. **C)** IN K236/240A PICs. **D)** IN R262/3/6 PICs. Images were taken at 100X magnification, each one representative of 30-50 permeabilized stained cells per field, four fields per slide, in four independent experiments.

This observation is consistent with previous reports of the karyophilic properties of the Vpr molecule itself [64, 184, 185], and the fact that the NLS mutant Vpr-GFP mutants remained in the cytosol suggests that the NLS activity in the integrase C-terminus may have hierarchical superiority over that conferred by Vpr or other constituents of the PIC.

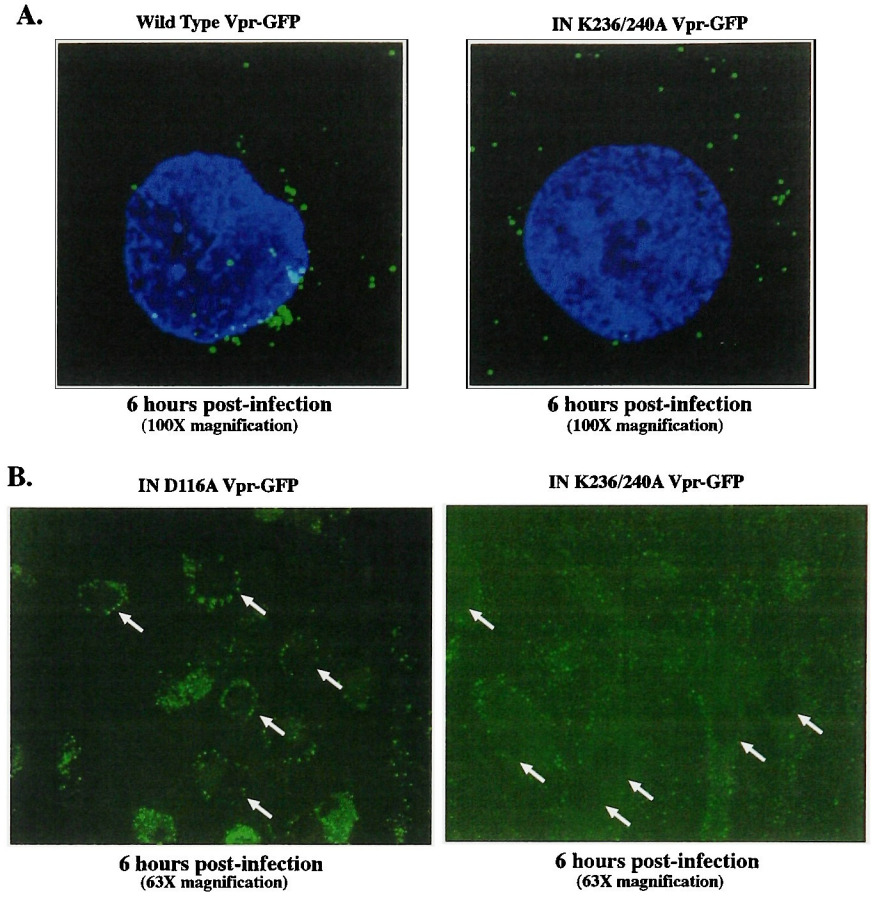


Figure 22. Vpr-GFP tagged IN NLS mutants display decreased nuclear localization in primary human macrophages. Vpr-GFP tagged virions were produced by double transfection of 293 T cells with a plasmid encoding the Vpr-GFP fusion protein (T. Hope) and a plasmid specifying an M-tropic Vpr-deficient HIV molecular clone (R73-Yu2-deltaVpr). Primary human macrophages were then infected with these Vpr-GFP viruses (+/- the IN K236/240A NLS mutation) at a high MOI, and cells were visualized at 6 hours post infection. **A)** Representative cells (of 50-100 per slide) are shown for WT and IN K236/240A Vpr-GFP infections with the nuclear DNA stained with DAPI (blue). Individual PIC and PIC clusters are visible in green. **B)** Still photos from live-cell imaging of HIV infection of macrophages. The nuclei in each field are indicated with yellow arrows and GFP-tagged virions are shown in green. The D116A active site mutant showed a nuclear localized phenotype than the K236/240A mutant, reinforcing the importance of an intact IN C-terminus to nuclear import. Images are representative of 30-50 Vpr-GFP positive cells per field, four fields per slide, in two independent experiments.

CHAPTER 4. STABILITY AND GENE EXPRESSION FROM UNINTEGRATED COVALENTLY-CLOSED CIRCULAR HIV-1 DNA

When the human immunodeficiency virus infects target cells HIV integrase catalyzes the 3' processing and strand transfer reactions that are necessary for viral replication. However, HIV Integrase-mediated insertion of the proviral DNA into host chromosomal DNA is not absolute. It has been known for over a decade that circular forms of viral DNA accumulate within cells following infection with wild type HIV *in vitro* [186]. Yet, the role of these viral DNA circles in the life cycle of the human immunodeficiency virus has only recently begun to be elucidated. Thus, understanding the potential role that these viral DNA circles may play in supporting HIV infection and latency in various cellular compartments is of key importance.

The accumulation of 1- and 2-LTR circles in monocyte-derived macrophages and T cell lines following HIV infection with wild type virus *in vitro* has been described [187]. Previous studies with integration-defective HIV molecular clones and more recent studies with first-generation diketo acid HIV integrase inhibitors have shown that 2-LTR circular forms of viral DNA preferentially accumulate in T cell lines during infection with a catalytic triad HIV integrase mutant virus or with wild type virus treated with HIV integrase

inhibitors [175]. These episomal circular viral DNAs are capable of limited *tat* and *nef* gene expression [41, 187]. However, viral p24 gag gene expression from 2-LTR circles has never been documented. Accordingly, infection with integration-defective catalytic triad mutant HIV molecular clones results in a single-cycle non-productive infection *in vitro*.

During a productive HIV infection, antigen presenting cells including dendritic cells and macrophages process viral protein antigens and present them to T cells to initiate the antiviral immune response [reviewed in [188]]. However, the ability of viral proteins expressed from 2-LTR circles to stimulate antiviral immune responses has not been ascertained. A recent report by Bakri et al. describe the detection of 1- and 2-LTR circles in cultures of immature and mature dendritic cells [189]. However, prior to the data presented in this thesis, there have been no published accounts of long-term persistence or protein expression from LTR circles from integration defective HIV viruses or HIV vectors in myeloid-derived cells such as primary human macrophages or immature dendritic cells.

I. HIV-1 2-LTR circles are stable in primary human macrophages and immature dendritic cells

Primary human macrophages were infected with wild type and D116A integrase mutant HIV viruses or lentiviral vectors and total genomic DNA was isolated at various times post infection. A diagram depicting how the D116A mutant lentiviral vector was produced by alteration of the packaging plasmid R8.91 (kind gift of D. Trono) used in a triple transfection of HEK 293T cells [Figure 23].

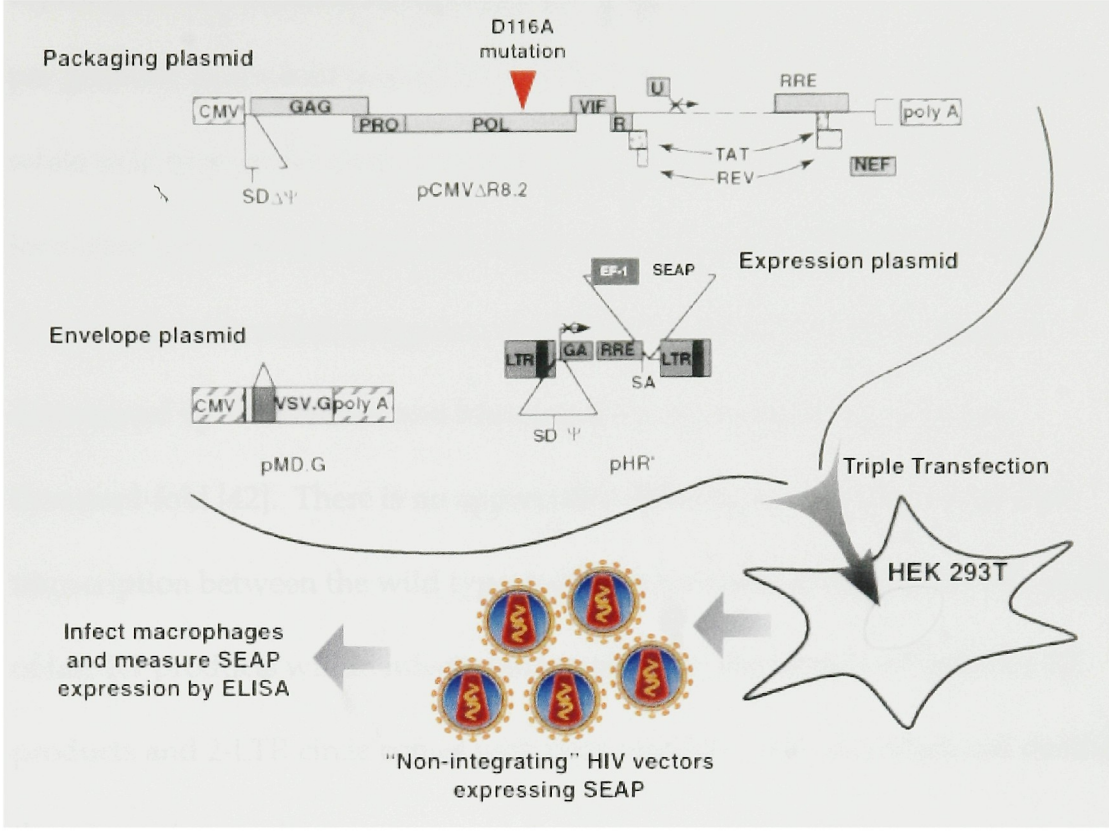


Figure 23. Production of integration-defective lentiviral vector stocks. The D116A mutation was introduced into the *pol* reading frame of the lentiviral vector packaging plasmid pCMV-R8.2 (kind gift of D. Trono) to make pCMV-R8.2-D116A. The lentiviral expression plasmid pHR' was then altered by the insertion of an expression cassette encoding the gene for Secreted Alkaline Phosphatase (SEAP) (Clontech, Inc.) under the control of the elongation factor 1 alpha (EF-1) promoter to make pHR'EF-1-SEAP. SEAP-expressing non-integrating lentiviral vector stocks were produced by triple transfection of the pCMV-R8.2-D116A plasmid along with the pHR'EF-1-SEAP and a plasmid expressing the envelope protein from vesticular stomatitis virus (pMD.G). These non-integrating lentiviral vectors were then used to transduce primary human macrophages and SEAP production was measured at various time points post-transduction.

As expected, the D116A integrase mutant virus produced more 2-LTR circle copies per cell compared to wild type HIV [Figure 24]. 2-LTR circle copy number per genomic equivalent peaked after 24 hours in D116A infected macrophages, while wild type circles peaked after 8 days. This difference in the rate of circle formation between wild type and integrase mutant viral infections may be due to the severity with which integration is disfavored with the D116A mutation, determined by Wiskerchen and Muesing to be by as much as five to ten thousand-fold [42]. There is no appreciable difference in the level of reverse transcription between the wild type and D116A viruses, evidenced by the parity of late RT products within infected macrophages. The decline of both late RT products and 2-LTR circle copies with time coincides with increased cell death in the macrophage cultures.

Primary human macrophages were infected with wild-type as well as integration-defective (IN D116A) HIV-1 molecular clones, and total genomic DNA was isolated from these cells at various times post-infection. We were able to detect 2-LTR circles in macrophages from day 1 and up to 32 days post-infection with the integration-defective HIV-1 viral species, but the circles were barely measurable in the wild type infected cells at the latest time point [Figure 24a]. By day 32, the total number of both wild type and D116A integrase mutant

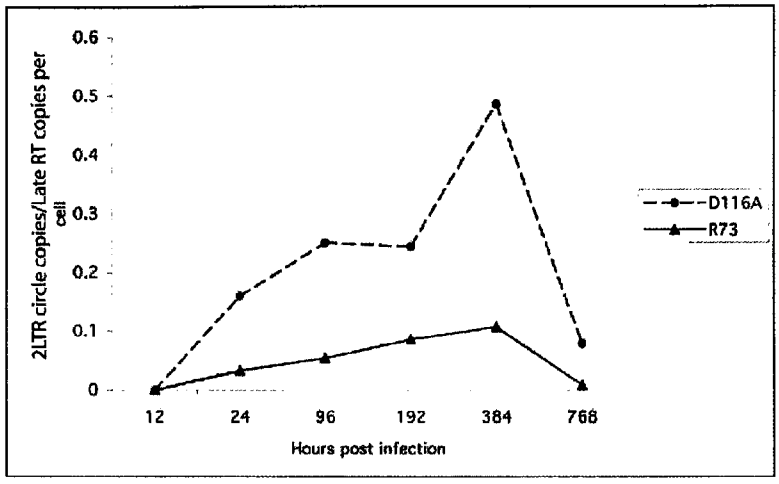


Figure 24a. HIV 2-LTR circles are stable in primary human macrophages. Primary human macrophages were infected with wild-type R7/3-Yu2 (triangles) and integration-defective D116A R7/3-Yu2 (circles) M-tropic HIV viruses (100ng p24/10^6 cells). Total genomic and extrachromosomal DNA was isolated from infected macrophages at days 1, 4, 8, 16, and 32 post-infection. One hundred nanograms of DNA from each sample was analyzed for 2-LTR circle content via Real Time PCR. Peak copy numbers of 2-LTR circles were reached by Day 16 post-infection, with a 3 to 10-fold difference in 2-LTR circle copies evident between the D116A IN mutant and the wild type R7/3 virus.

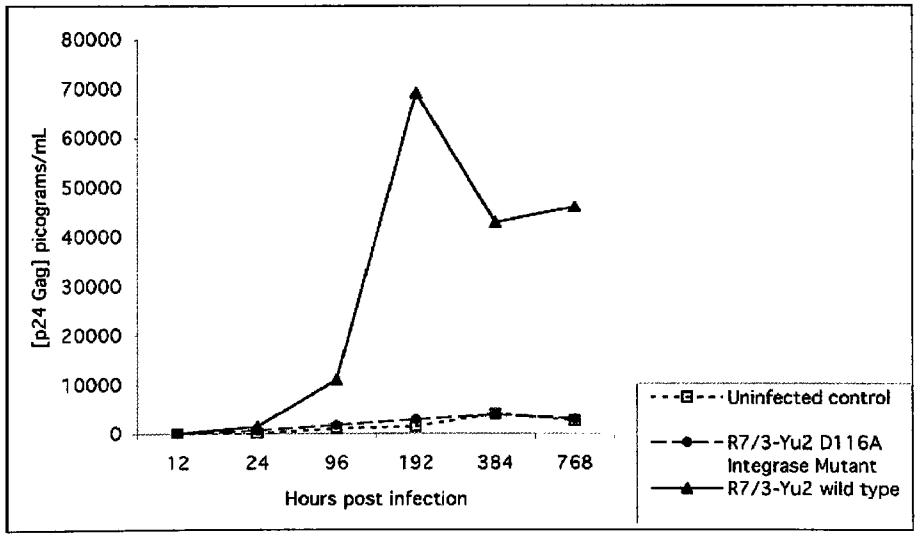


Figure 24b. p24 Gag expression in primary human macrophages following infection with wild type R7/3-Yu2 eGFP and D116A R7/3-Yu2 eGFP. Media samples were taken at the various time points (as above) and analyzed via ELISA assay for p24 Gag expression. The D116A mutant did not produce detectable levels of HIV gag, suggesting that LTR circles alone are incapable of sustaining a viral infection.

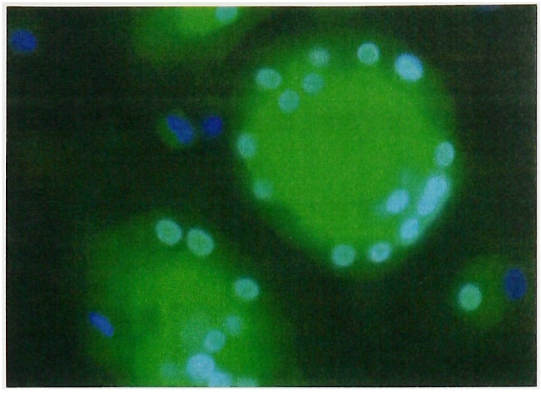
2-LTR circles decreased from peak levels (at day 16), which appears to correlate with the gradual disappearance of cells due to cell death. While we consistently observe a 10-fold elevation in 2-LTR circle copy number upon infection with the D116A mutant virus, we were unable to detect viral p24 Gag expression from macrophage cultures infected with the D116A integrase mutant virus [Figure 24b]. Thus, our data indicate that 2-LTR circles are stable for at least one month in macrophages following infection with integration-defective HIV and they appear to persist throughout the life of the cell. In similar experiments, primary monocyte-derived immature dendritic cells were prepared as previously described [190] and infected with wild type or D116A mutant HIV on day 6 of the cultures. Infected cells were washed and total genomic DNA was isolated at two time points at day 2 and 14 post-infection. Quantification of 2-LTR circles present in the total genomic DNA samples from day 2 and Day 14 shows that the circles persist in immature dendritic cells through Day 14 post-infection. The D116A mutation produced a similar increase in circle copy numbers compared to wild type controls as was seen in macrophage infections using these same viruses.

II. 2-LTR circles express low levels of enhanced green fluorescent protein (eGFP) in macrophages

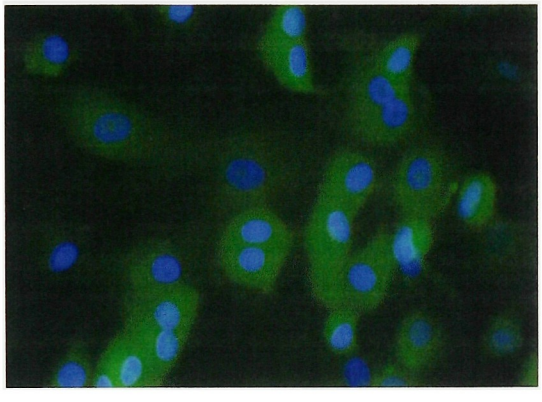
Low levels of viral gene expression were evident from unmodified 2-LTR circles. Consistent with previous results, we were unable to detect viral p24 Gag expression from macrophage cultures infected with the D116A mutant version of R7/3, an HXB-2-derived proviral subclone of HIV-1 [191]. Similarly, macrophages infected with an integration defective macrophage-tropic R7/3 variant, R7/3-Yu2-eGFP, which expresses the enhanced green fluorescent protein gene from the *nef* position, displayed markedly decreased levels of eGFP compared to wild type controls [Figure 25]. The reasons for this lack of robust gene expression from unintegrated viral DNA templates is unclear, and may be caused by a variety of factors, including the supercoiled nature of the circularized DNA, the proximity of circles to transcriptional machinery in the nucleus, as well as the relative effectiveness of the viral 5′ LTR promoter when the HIV genome is in circular form. Indeed, the presence of two LTRs side by side in the circularized viral DNA may cause promoter occlusion as an overload of transcription factors and other molecules that bind to the sequences in the LTRs could prevent the proper initiation of RNA polymerase II-mediated elongation.

III. Integration-defective VSV-G-pseudotyped lentiviral vectors express measurable levels of secreted alkaline phosphatase (SEAP) in macrophages

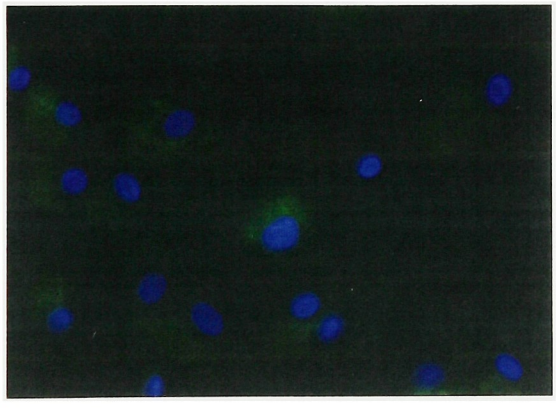
In order to determine whether we could overcome possible negative effects on the 5'LTR promoter in 2-LTR circles and thus increase the level of gene expression from them, we constructed a series of lentiviral vectors that contained an internal promoter, EF-1a (elongation factor 1a), positioned downstream of the LTR in the circularized viral DNA. The vectors were made integration defective by way of a triple mutation of the catalytic residues (DDE motif, D64A, D116A, E152A) [42] in the active site of the lentiviral integrase and addition of these vector stocks to primary human macrophages resulted in non-productive, single-cycle infection with a characteristic increase of stable, unintegrated covalently closed circular viral DNA. We inserted the gene for Secreted Alkaline Phosphatase (SEAP) downstream of the EF-1a promoter in order to serve as a reporter for circle-driven gene expression. Following infection with EF-1a-SEAP 2-LTR circle vectors, cells were washed thoroughly to remove non attached virions from their surface, fresh media was added, and the cells were maintained in culture for an additional seven days.



R73-Yu2-eGFP
wild type



D116A IN mutant



Uninfected Control

Figure 25. HIV 2-LTR circles express low levels of enhanced green fluorescent protein (eGFP) in macrophages. Macrophages at day 21 following infection with either M-tropic wild type R7/3-Yu2-eGFP (panel A), D116A -Yu2-eGFP integrase mutant HIV viruses (panel B), or mock infected (panel C) are shown at 40X magnification. The D116A IN mutant displayed low levels of eGFP expression when compared to wild type controls, suggesting that 2-LTR circles are capable of limited gene expression in primary APC. The data shown is representative of three independent experiments.

Analysis of infected cell supernatants using an ELISA based assay to detect SEAP, revealed that the insertion of a promoter (EF-1a) internal to the 5' LTR resulted LTR circle gene expression levels that were 25% of a wild type, integration-competent vector with the analogous promoter/reporter combination [Figure 26]. SEAP gene expression from the 2-LTR circle vectors peaked at 4 days, and did not decrease appreciably throughout the duration of the culture (8 days). The presence of 2-LTR circles and SEAP had no apparent deleterious effects on cell viability in the infected cultures as determined by trypan blue exclusion. This data suggests that, with the proper promoters, 2-LTR circle gene expression can be moderately enhanced, and such modified lentiviral vectors may represent a novel platform for gene therapy in non-dividing cells like macrophages and imDC.

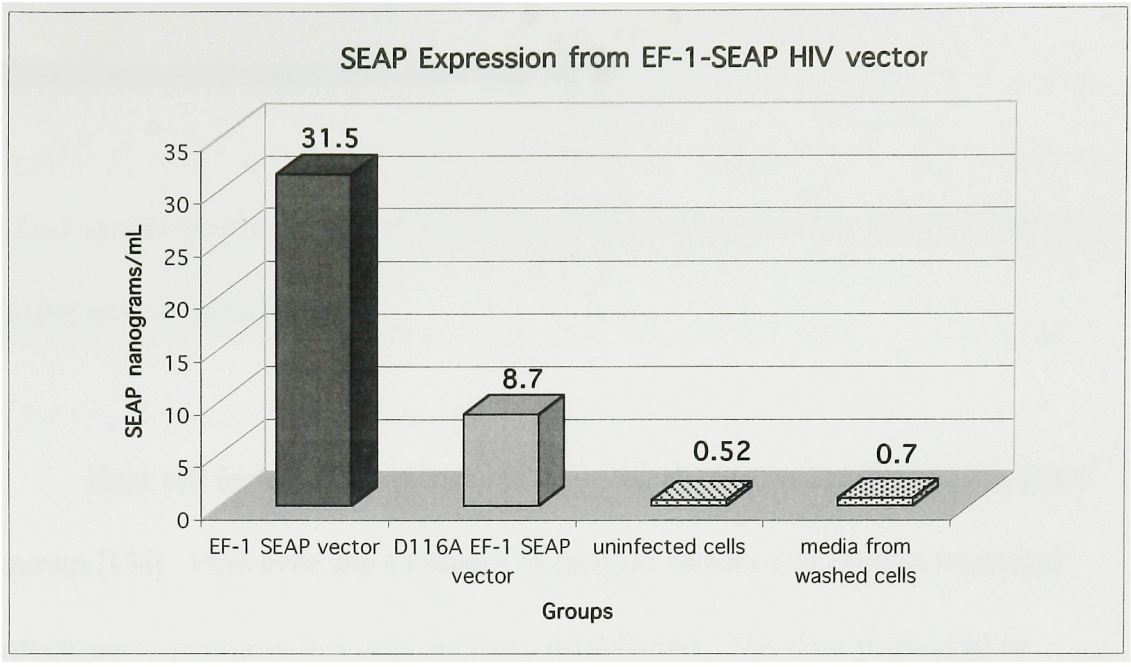


Figure 26. Integration-defective VSV-g-pseudotyped lentiviral vectors express measurable levels of secreted alkaline phosphatase (SEAP) in macrophages. Lentiviral vectors were engineered to be integration-defective by the introduction of an active site mutation (D116A) to the integrase portion of the *pol* reading frame in the packaging plasmid. The vectors were designed to express SEAP under the control of the elongation factor 1a promoter (EF-1), pseudotyped with the vesticular stomatitis virus envelope glycoprotein (VSV-g), and were then used to transduce primary human macrophages at an MOI of 10. After a 12 hour incubation with vector, cells were washed and fresh media was replaced in each well. Media was then collected at 4 days post transduction and analysed for SEAP expression by a chemiluminescent detection assay. Media from the washed cells as well as uninfected cells are included as controls. The data shown is representative of two separate experiments.

CHAPTER 5. SUMMARY AND CONCLUSIONS

I. Lv-1 species-specific lentivirus restriction occurs at an early stage of the HIV life cycle during reverse transcription

Host cell factors that restrict murine retroviral infections have long been known [154]. However, the existence of cellular factors that restrict lentiviral infections in primates has only recently established. The data presented in Chapter 2 of this thesis and in published form [149] represents the first definitive example of a host cell activity that restricts the tropism of both HIV and SIV in monkey and human cells. Analysis of viral DNA intermediates following infection of both permissive and non-permissive primate cells revealed a striking replication defect for incoming virions at the step of early reverse transcription, which carried through to subsequent steps of late reverse transcription, and presumably affects nuclear import of restricted viral cores [Figure 11]. This host cell factor was originally called Lv1 (lentivirus susceptibility factor 1), but subsequent studies have identified Lv1 as the cellular molecule TRIM5-α [165, 166]. Indeed, figure 2 of the report by Stremlau and colleagues describing the identification of TRIM5-α alpha as the lentivirus susceptibility factor shows a defect in early reverse transcription for incoming HIV virions in rhesus macaque

cells containing high levels of rhesus TRIM5-α that is analogous to the defect we observed in our initial experiments with Rh.F cells (also derived from macaque monkeys). Thus, the results of our initial studies, in conjunction with supporting reports from others, has now firmly established that the Lv1/TRIM5-α host cell restriction factor exerts its effects at the early stages of viral life cycle, at a step following entry and prior to reverse transcription.

As mutations in the CA proteins of the incoming virions, as well as the addition of saturating amounts of lentiviral particles can overcome the Lv1/TRIM5-α restriction [149, 192], the most likely mechanism of action for this factor is to bind to incoming virions in a manner that prevents CA disruption and subsequent uncoating or the downstream formation of reverse transcription complexes and functional PICs. While this proposed mechanism of action for Lv1/TRIM5-α has not yet been proven, several recent observations support this assertion. First, the binding of the cellular protein Cycolphilin A (CypA) to HIV capsids has been shown to protect incoming virions from the effects of Lv1/TRIM5-α in vitro [193]. Second, the CypA binding site in the CA protein overlaps an important determinant of Lv1/TRIM5-α restriction-factor recognition in humans and nonhuman primates [193, 194]. Notably, the CA–CypA interaction is required for restriction of HIV-1 by certain nonhuman primates, but in humans the CA–CypA interaction facilitates HIV-1 infection,

presumably because it at least partially inhibits restriction factor recognition[193]. Thus, the preponderance of evidence points to the lentiviral CA protein as the main target for host cell restriction factors like Lv1/TRIM5-α.

An interesting recent observation of lentiviral restriction in New World monkeys, such as the owl monkey (*aotus trivirgatus*), demonstrates the lengths to which some host species go to generate host cell restriction factors to prevent lentivirus infection. Sayah and colleagues [195] followed up on a previously paradoxical observation that CypA-deficient HIV virions were able to overcome the block to HIV infection in owl monkey (OMK) cells. Normally, CypA incorporation into virions enhances their infectivity [reviewed in [164, 196-200] (presumably by protecting them from the binding of restriction factors, as described above), so the result in OMK cells was surprising, and compounded by the fact that reintroduction of CypA into these cells following CypA-specific RNA knockdown by RNAi failed to restore the antiviral activity [195]. Instead it was discovered that a hybrid molecule of TRIM5-α and Cyclophilin, termed TRIMCyp, had arisen in owl monkeys via retrotransposon-mediated insertion of a cDNA containing CypA into the TRIM5-α locus in the owl monkey genome as they diverged from old world primates [195]. This molecule, and not the traditional TRIM5-α found in macaques (no TRIM5-isoform has yet been identified in owl mokeys), is believed to be responsible for the species specific

restriction of lentiviruses in these monkeys. Whether the exon shuffling that produced TRIMCyp was purely serendipitous, or the result of evolutionary pressure exerted by the hyper-infection of ancestral owl monkey populations with pathogenic lentiviruses to which they were previously susceptible remains unclear. Undoubtedly, future studies in this area will uncover some of the molecular intricacies that vertebrates use to outmaneuver the pathogens that infect them.

II. 2-LTR circles are stable in primary human macrophages and may be a platform for gene therapy based vaccines

During Human Immunodeficiency Virus-type 1 (HIV-1) infection, the viral integrase catalyzes a 3′ OH processing reaction that serves to activate the linear viral DNA termini for a subsequent strand-transfer reaction in which viral DNA is covalently joined to the host chromosome. Sustained viral replication absolutely requires the integration of the viral DNA since full transcription competency is achieved only after the creation of the proviral state. However, when the catalytic activity of integrase is compromised by small molecule

inhibition or via specific point mutations in the catalytic core domain of the enzyme, unintegrated viral DNAs accumulate in the nucleus in the form of 1-LTR and 2-LTR covalently closed circles [42, 175]. These viral LTR circles were believed previously to be labile byproducts of HIV infection[186], but our work as well as other recent studies [41, 201] has shown that they are inherently capable of limited expression of certain viral genes, namely *tat* and *nef* (as evidenced by the expression of our in-frame nef-eGFP fusions shown in Figure 25). However, for reasons unknown, we were unable to demonstrate robust viral gene expression from unintegrated circular viral DNA, and integration defective viruses never produce detectable levels p24 gag expression within infected macrophages. The inability of 2-LTR circles to produce enough viral protein to sustain a persistent infection may be due to the inability of the major viral promoter, the 5'LTR, to function outside of the context of an integrated provirus. While other possible reasons for the reduction in gene expression from these circles, such as the level of DNA supercoiling and the proximity to transcriptional machinery, cannot be ruled out entirely, the results of our experiments with the EF-1a-SEAP lentiviral vector system [Figure 26] suggest that the identity and/or conformation of promoter used to drive gene expression from 2-LTR circles is an important determinant of the amount of transcription they can support.

As the 2-LTR circles can easily be detected and quantified by real time PCR in cultures of macrophages and T cells, numerous groups have sought to use them as an indicator of recent HIV infection in order to glimpse the early moments of the establishment of viral latency in these cells [202]. A recent report by Sharkey et al. describing the persistence of episomal HIV intermediates in the PBMCs of patients on highly active anti-retroviral therapy proposed that 2-LTR circles can serve as a reliable marker of recently infected cells [203] . However, our data showing long-term survival of 2-LTR circles in macrophage cultures, as well as recent experiments from other groups showing the same in T cell lines, refute this assertion [204]. Similarly, in SupT1 and MT-4 T cell lines the slope of the rate of decay of 2-LTR circles is very similar to the slope of the rate of cell division, suggesting that the decline of 2-LTR circles is primarily due to dilution during cell growth instead of rapid decay upon recent infection [204]. In addition, a recent study by Butler et al. showed that 2-LTR circles peaked in abundance at 24 hours and persisted indefinitely in aphidicolin growth-arrested SupT1 cells, noting that declines in 2-LTR copy numbers in these cultures was due to the death of infected cells [174, 205]. This result is similar to what we observe in thirty-day macrophage cultures wherein 2-LTR circle copy numbers decrease over time, apparently due to cell death. As these episomal viral byproducts display a propensity to persist indefinitely inside of infected cells,

and it would be difficult to separate cells that had been recently infected with HIV from those that continuously harbored virus, we believe that measurment of 2-LTR circles would not be a reliable marker of recent HIV infection *in vivo*.

The data presented in this thesis indicates that 2-LTR circles persist until the point of cell death in macrophages, following infection with wild type or integration defective HIV. Using sensitive molecular beacon-based real time PCR assays, we have tracked the appearance of viral DNA intermediates, including early and late reverse transcription products, as well as 2-LTR circles at various times post-infection. In addition, we monitored the capacity of integration-defective HIV viruses to express the viral p24 Gag protein in antigen presenting cells. We were able to enhance the level of gene expression to one-fourth that of wild type by placing a defined promoter-driven transcriptional unit (EF-1a-SEAP cassette) in a location that would be internal with respect to the 5'LTR once the 2-LTR circles form in the nucleus. As these experiments were performed in a non-dividing antigen presenting cell (macrophages), these results suggest that lentiviral vectors modified to form 2-LTR circles may be worth exploring in gene therapy settings. The non-toxic nature and persistence of such gene expression episomes would avoid some of the pitfalls that have been seen with other retrovirus-based gene therapy vectors, such as the insertional mutagenesis which

lead to lymphoma in patients treated with MLV vectors used in human clinical trials for severe combined immunodeficiency syndrome [206-210]. Indeed, if a sufficient level of continuous HIV gene expression from stable 2-LTR circle templates is capable of priming anti-viral immune responses, then HIV integrase inhibitors might have expanded immunological benefits in addition to their ability to lower viral load. In addition, if additional alterations to 2-LTR circle architecture (such as the inclusion of elements, like scaffold attachment regions, that link them to areas of active gene transcription in the nucleus), can further increase in their capacity to express genes, then non-integrating lentiviral vector systems may become the platform of choice for gene therapy applications in the future.

III. The carboxyl terminus of HIV-1 integrase specifies a transferable nuclear localization signal (IN NLS) that functions in primary cells

In previous studies, systematic mutagenic analysis was employed to identify a potent nuclear localization signal (NLS) within the carboxyl-terminal domain of HIV-1 integrase [167]. A region was identified spanning the charged-clusters of basic amino acids at residues 236/240 and 262/263/266 and overlapping with a previously defined SH3 structural motif [168, 211, 212] that

had profound effects on HIV IN import in the context of IN-eGFP fusion proteins. In contrast to several earlier studies by others, we employed a novel, codon-optimized version of HIV-1 integrase that enabled us to observe the effects of NLS mutations on the nuclear accumulation of the *native* integrase protein. We further demonstrated that the carboxyl-terminal NLS is transferable—when fused in a variety of configurations to proteins that are otherwise cytoplasmic it confers nuclear localization to the chimeric molecules. Importantly, this transferable NLS was functional in non-dividing cells that support HIV infection *in vivo*, such as immature dendritic cells and CD8-depleted (CD4+) peripheral blood lymphocytes. That the IN NLS mutations prevented nuclear import of large cargo proteins in non-dividing cells minimizes the possibility that they simply coordinate integrase binding to chromosomal DNA.

Virions incorporating NLS mutations are intact with respect to integrase catalytic function as well as to other biochemical parameters. However and more importantly, a quantitative analysis of viral DNA intermediates formed during infection of primary lymphocytes and macrophages (cell-types critical for the propagation of HIV-1 *in vivo*) revealed a profound two- to three- log defect specific to the nuclear import of mutant viral DNA. In addition, when these IN NLS mutants were used to make fluorescently tagged Vpr-GFP virions, live cell imaging of macrophage infections with these viruses reinforced the results

obtained in the real time PCR and eGFP-MBP nucleofection experiments, as a nuclear excluded phenotype was observed in cells infected with the IN NLS mutant virus compared to the wild type IN NLS virus.

Interestingly, unlike canonical nuclear localization signals, the IN NLS activity cannot be transferred as a small peptide unit but instead requires the region of the protein spanning residues 220-270 [167]. When this region of HIV integrase is dimerized in solution, each monomer adopts an SH3-like fold [211, 213]. In this configuration, a highly charged surface is created at the solvent interface by the contribution of the basic residues at positions 236/240 and 262/263/266, each cluster contributed by a separate monomeric unit. Thus, orientation of the constituent residues of the IN NLS within the context of a larger three-dimensional space may be critical to its function as a facilitator of viral nuclear import. Although SH3 domains are not generally thought to interact with the nuclear transport machinery, at least one example of this type of interaction has been described. The SH3 domain of the human T-cell tyrosine kinase, Itk, has been reported to interact directly with a karyopherin-α (Rch1α) *in vivo* and this interaction is necessary and sufficient for T-cell receptor signal-regulated nuclear transport of the Itk protein [214, 215]. The fact that our mutants are defective in dividing as well as non-diving cells is concordant with recent data indicating that nuclear translocation of the HIV-1 genome is independent of

mitotic nuclear disassembly [216]. In fact, these studies reveal that the nuclear transport of viral DNA is actually delayed during as the cell undergoes mitosis [216], a result previously unanticipated and widely thought to be counterintuitive, as the nuclear membrane would be broken down during mitosis and the virus should have free access to the nucleus under these circumstances.

Similar to many previously identified NLS sequences, the residues which contribute to the integrase NLS are contiguous with, but distinct from, specific residues important for DNA binding. Although basic residues are critical for NLS activity, there is little homology to classic, bipartite, or basic NLSs such as that of nucleoplasmin (NP) and a karyophilic binding partner for integrase has not yet been identified. In previous reports, Gallay and colleagues stated that HIV-1 IN is brought to the nuclear pore through the interactions of karyopherin-alpha with karyopherin-beta [217, 218]. However observations reported by Depienne et al. [219] suggest that IN can dock at the nuclear pore and enter the nucleus in their absence of these nuclear transport receptors. The basis for this discrepancy remains to be determined, but is likely attributed to the difference in reagents (specifically the type recombinant integrase proteins) used in the various studies, or the isotype of the karyopherins and other reagents used in the nuclear import

assays. For example, the report by Depienne et al. relied upon a purified integrase protein conjugated to a fluorescent molecule (Cy3). Their observation that this fluorescently conjugated IN molecule can localize to the nucleus in the absence of cytosolic factors (including the karyopherins) does not necessarily prove their conclusion that IN mediated nuclear import is karyopherin-independent because the addition of the Cy3 moiety could affect IN oligomerization, allowing it to passively diffuse through the NPC as a single molecule (32kD, below the NPC exclusion limit) where it binds to DNA and appears nuclear, or otherwise influence the trafficking of the native IN molecule. In contrast to the Depienne et al. report, our results with the eGFP-MBP fusion proteins suggest that the C-terminus of integrase must interact in some manner with the host nuclear import machinery in order to carry this large protein cargo through the NPC.

Additional support for the notion that integrase mediated nuclear import involves host cells factors is found in three recent publications describing host proteins that interact with HIV-1 integrase. These molecules include LEDGF [220], importin7 [221] and EED [222] all of which have biochemical and cytological characteristics that certainly warrant further investigation. LEDGF and Importin7, have been shown to interact with HIV-1 integrase in a manner that is consistent with either a role in the chromosomal targeting of the integrase

protein [220], or a function in the nuclear import of the viral RT complex itself [221]. In the case of LEDGF, this interaction is dependent upon the amino-terminal and core domains of integrase, as the carboxyl-terminal domain is reportedly dispensable for this interaction *in vivo* [220, 223]. However, our data indicates that the IN C-terminal domain alone is both necessary and sufficient for localization to the nucleus. A plausible explanation to reconcile these apparently disparate results is that LEDGF simply anchors HIV-1 integrase to host chromosomes after nuclear import has occurred, as the size of the HIV IN amino-terminal+core domain segment used in their experiments is roughly 23 kD, well below the 60 kD exclusion limit of the nuclear pore.

Importin7 (an isoform of karyopherin α) acts as a heterodimer with importin-β (karyopherin β) to facilitate the nuclear import of histone H1 [224], certain ribosomal proteins [225, 226], as well as the RT complexes associated with genomic HIV-1 import in permeabilized cells [221]. Although importin7 has been reported to interact with the HIV-1 integrase protein *in vitro,* the experiments performed to demonstrate this interaction required the addition of HeLa cell cytoplasmic extract to the system in order to be detected [221]. Therefore, it is possible that the contribution made by importin7 to nuclear import of RT complexes may not result from its direct interaction with integrase *per se,* but is instead coordinated through a complex of importin7/karyopherin β, integrase,

and some other as-yet-unidentified "bridging" molecule(s) in the cytosolic extract. A good candidate for such a potential bridging molecule the is human embryonic ectoderm development (EED) protein, which has been shown to bind integrase *in vitro* [222]. Interestingly, EED also binds other HIV proteins, namely Matrix and Nef [227, 228], suggesting that association with this cellular protein may provide the virus with a replicative advantage.

EED belongs to the polycomb group of proteins (PcG) [229] that were originally identified in *Drosophila melanogaster* as nuclear proteins containing multiple copies of the WD-40 repeat [230] that act to remodel chromatin by altering the accessibility of DNA to factors required for gene transcription [231]. Homologues of these proteins are highly conserved in mammals [232]. Significantly and quite unexpectedly (as integrase's toxicity to yeast cells has confounded earlier attempts to define its binding partners) the EED protein has been shown to interact strongly with the HIV-1 integrase protein in the context of a yeast two-hybrid system [222]. This interaction appears specific for the carboxyl-terminal domain of integrase. Perhaps most interestingly, the specific binding between integrase and EED can be competed against *in vitro* with recombinant bacteriophage that display peptide segments that map to the identical set of charged-cluster amino acid residues that we have identified as critical to integrase NLS function *in vivo*.

In humans, a molecule that is homologous to drosophila EED in both DNA and amino acid sequence, has been described independently as a protein that binds to the cytoplasmic domain of the integrin beta-7 subunit, alpha-4, beta -7 [233]. This protein, designated WAIT-1 (WD-40 repeat protein Activated In T-Cells) was initially characterized in circulating lymphocytes where it appears to be involved in the transduction of signals arising from the integrin beta-7 receptor [233]. In lymphocytes, signals arising from the integrin receptor are thought to be transmitted via EED/WAIT-1 from the inner plasma membrane into the nuclear compartment to affect regulation of inducible gene activity [233]. As would be expected for a protein involved in control of transcription, EED/ WAIT-1 is localized predominately to the nucleus in cycling cells as determined by its fusion to a fluorescent indicator protein [234]. Thus, EED/WAIT-1 would provide all of the essential features that incoming PIC, and the integrase C-terminal NLS contained within it, might desire in potential a binding partner, including a propensity to rapidly localize to the nucleus in activated T cells, the natural target cells for HIV infection *in vivo*.

Structurally, EED/WAIT-1 displays some features that would support its potential role as a binding partner for our IN NLS during viral nuclear translocation. Of particular interest is the proline-rich N-terminal region of the proteins which harbors two closely spaced PXXP motifs—a motif commonly

found in inter- or intra-molecular interactions with SH3 domains [235]. Interestingly, prolines at $P^{60}XXP^{63}$ in EED/WAIT-1 exist in the context of a sequence known for other proteins to be utilized to coordinate SH3 domain binding [236]. Finally, EED/WAIT-1 has been demonstrated to associate rapidly with regions of the nuclear membrane near the nuclear pore complex following infection with HIV-1 [222]. Importantly, integrase co-localizes with EED/WAIT-1 at the pore peaking at 6 hours post-infection, consistent with our results showing that this same time frame is sufficient for nuclear localization of our Vpr-GFP tagged viruses [see Figure 22] [222]. Future studies of HIV nuclear import should seek to clarify the interactions between sequence motifs such as the C-terminal NLS in HIV IN described in this work, and soluble cellular factors such as EED/WAIT-1 and karyophilic molecules like importin7. Generating a detailed understanding of the molecular events and sequence motifs that support HIV's specific nuclear import pathway will be vital to our ability to discover novel antiviral compounds that block this critical step of the early viral life cycle.

FUTURE STUDIES:

I. Ligand blot methodology may reveal interactions between HIV integrase and host factor(s) that facilitate viral nuclear import

Nuclear pore complexes can be isolated by biochemical means and subjected to two-dimensional gel electrophoresis under denaturing conditions. This results in a characteristic banding pattern of nuclear pore proteins that can be transferred to nitrocellulose and probed by standard Western blotting techniques. In order to discern whether a given recombinant protein or multiprotein complex (i.e. an HIV PIC) is capable of binding to nuclear pore proteins, one can use a solution of that protein in place of the primary antibody to probe nuclear pore blots in a technique called ligand blotting [237, 238]. The recombinant protein or complex could be used in isolation or in the presence of specific cytosolic extracts containing requisite accessory factors (such as karyopherins or other adaptor molecules) that facilitate nuclear pore protein binding. This is followed by detection of the protein in question, in this case HIV integrase, with specific monoclonal antibodies, such as the mouse monoclonal antibody 6G5, that is specific for the N-terminus of the enzyme. By using recombinant HIV-1 integrase and PICs (both wild type and NLS mutant) on such nuclear pore ligand blots in the presence of cytosolic extracts from various primary cell types that naturally support HIV infection including activated CD4+

T lymphocytes macrophages we plan assess which nuclear pore components bind HIV integrase *in vitro*. An example of a preliminary experiment using ligand blot methodology with wild type and NLS mutant HIV PICs is shown in Figure 27. It appears that HIV integrase binds to numerous constituent proteins in the rat liver nuclear pore complex including the lamins, histones, and possibly some of the smaller nuceloporins. Future experiments are envisioned in which varying concentrations of suspected integrase-binding adaptor molecules, such as EED and LEDGF, as well as other viral proteins thought to be involved in nuclear import (i.e. MA, Vpr), are titrated onto ligand blots to determine the extent of their contribution to nuclear pore binding.

II. PIC immunoprecipitation may reveal differences in wild type and NLS mutant virus interactions with FG nucleoporins in the NPC

Extraction of rat liver nuclei with the zwitterionic detergent, empigen, results in an enrichment of some of the higher molecular weight constituents of the nuclear pore complex. Large FG nucleoproins like Nup358, Nup214, and Nup153 are present in these empigen extracts, along with Nup62 and other FG nups [83]. When purified cytosolic extracts from HIV infected macrophages are added to empigen extracted rat liver nuclei, it may be possible to discern differences

between wild type and NLS mutant virions with respect to their ability to bind to FG nups by attempting to immunoprecipitate bound FG nups with antibodies specific for viral proteins, such as the anti-IN N-terminus monoclonal antibody 6G5. A diagram decribing the reparation of HIV pre-integration complexes (PICs) is shown in Figure 28.

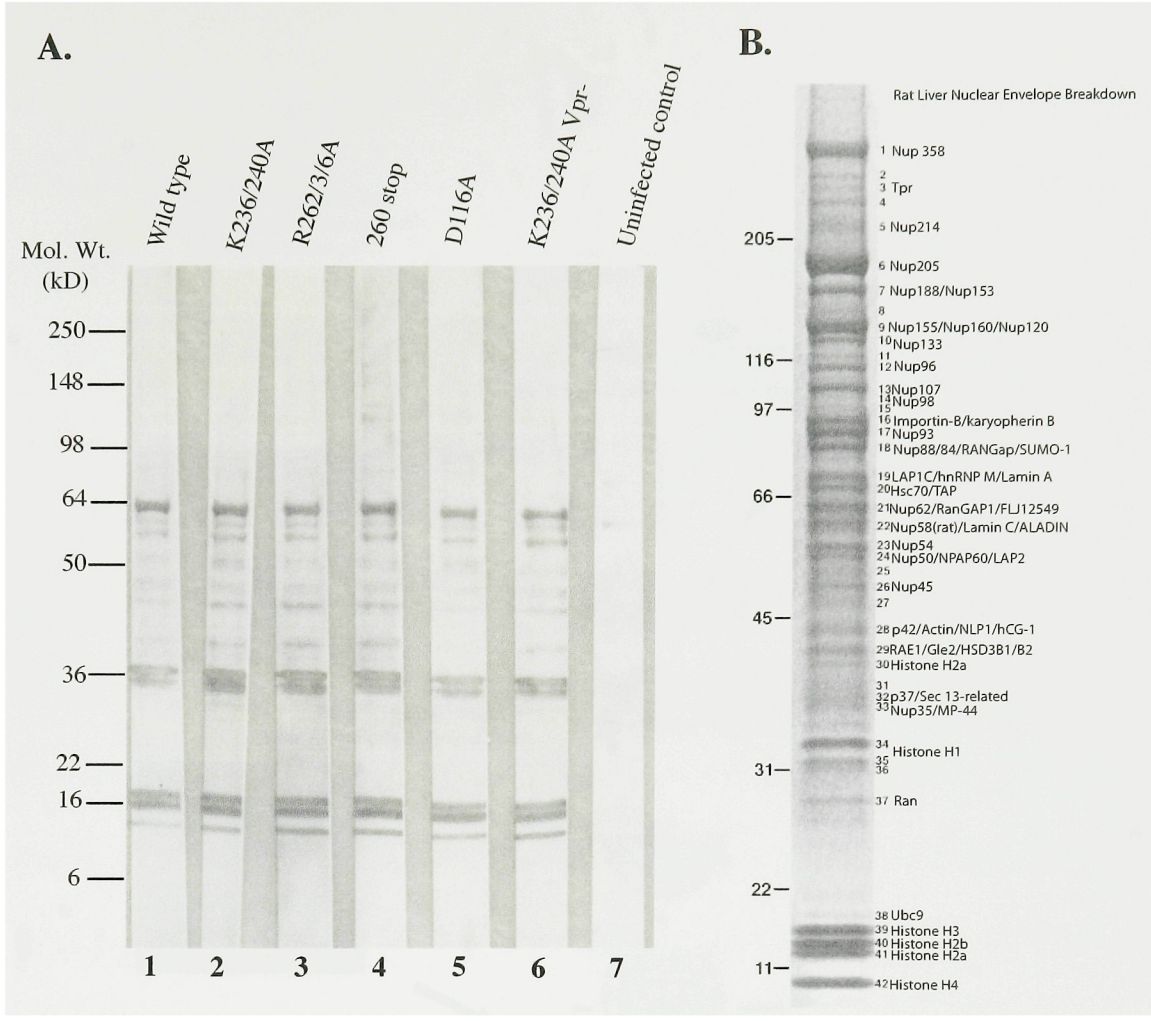


Figure 27. Ligand blotting of rat liver Nuclear Pore Complexes with WT and NLS mutant PICs. Rat liver nuclei were isolated, detergent solubilized, and run on a 4-20% Tris-Glycine gel (2 units per well). Nuclear proteins including lamins, histones, and nucleoporins were transferred to a nitrocellulose membrane by Western blotting and the blot was cut into strips well by well. Each strip was placed in a well of an 8-well tray, blocked with 3% non-fat dry milk, and incubated for 2 hrs at room temp with purified PICs from wild type and NLS mutant infected macrophages. A monoclonal antibody to the N-terminus of IN was then added (mAb6G5) and detected with an anti-mouse HRP-conjugated secondary antibody. Cytosolic extract from uninfected macrophages was included as a control under the same staining conditions (lane 7). **A)** Ligand blot of rat liver NPC with HIV PICs reveals numerous nuclear proteins that are reactive with HIV integrase. **B)** A stained blot of rat liver NPC with bands that have been identified by mass spectometry [ref 119]. Comparison with the ligand blot suggests that HIV IN in the context of PICs binds to histones (H2b, H2a, H1, and H4), lamins (lamin C), and perhaps some smaller nucleoporins (Nup54, Nup45, and p42). Data are representative of two independent experiments.

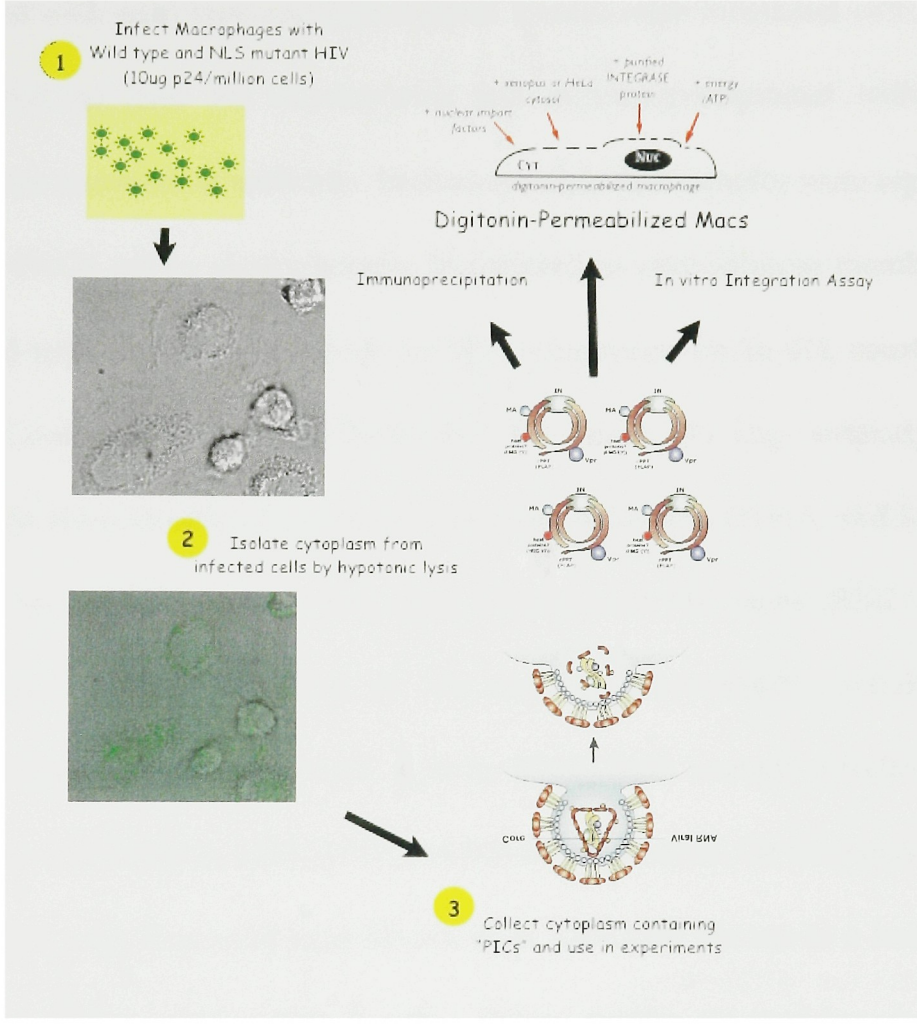


Figure 28. Preparation of pre-integration complexes (PICs) from HIV-infected macrophages. Primary human macrophages were infected with high amounts of HIV (MOI of 10,000), and infection was allowed to proceed for 6 hours at 37°C, to allow reverse transcription and subsequent PIC complex formation. After 6 hours, infected cells were washed twice with hypotonic lysis buffer, then incubated in ice-cold hypotonic lysis buffer with 50μg/mL digitonin detergent. Lysed cells were physically disrupted by passaging through a 25-gauge blunt needle and syringe, then cytosolic and nuclear extracts were made according to the protocol found in the Materials & Methods section. Cytosolic extracts were then snap frozen in liquid nitrogen then stored at -80°C until use in digitonin-permeabilized cell assays.

Figure 29 shows an example of an experiment in which PICs from cells infected with wild type and NLS mutant viruses were incubated with empigen-extracted rat liver FG nuceloporins and immunoprecipitated with an anti-integrase monoclonal antibody. Immunoprecipitated samples were separated on a SDS-PAGE gel by electophoresis, transferred to nitrocellulose membrane, and probed with an antibody specific for FG nucleoporins (mAb-414, courtesy of Dr. Elias Coutavas, Blobel lab, Rockefeller U.) Small, FG nup antibody reactive proteins are noticeable in the lanes containing wild type, D116A, and K236/240A PICs that appear absent in the other NLS mutant PIC lanes (R262/3/6A and 260stop). These bands may be the result of a proteolytic activity that is specifically active during wild type or K236/240A mutant infections that is absent or inhibited in R262/3/6A and 260 stop infections. Or, they may represent small FG nups that wild type virions (and K236/240 virions to a lesser extent) bind to during viral nuclear import. Future studies are envisioned using this PIC-empigen-extracted NPC immunoprecipitation assay in the presence of different molecules, such as recombinant EED (or PXXP mutants of EED, incapable of binding to HIV IN), as well as recombinant importin7 to reveal the complexes interactions that likely occur between HIV PICs and the FG nucleoporins during viral nuclear import.

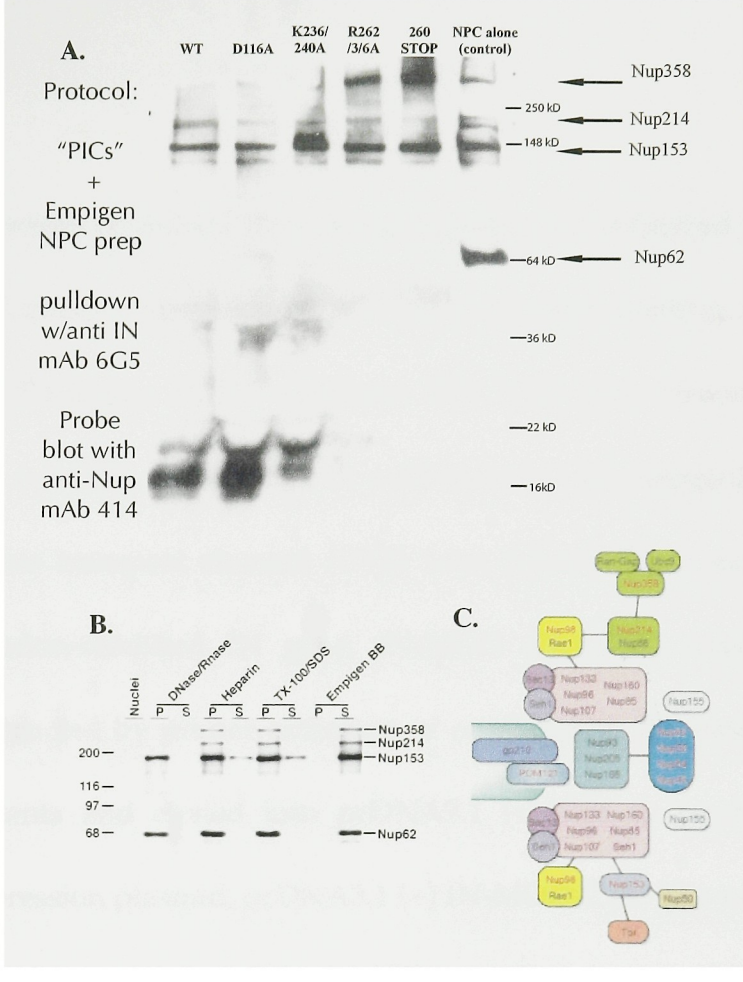


Figure 29. Immunoprecipitations with empigen-purified NPC and HIV PICs. Rat liver nuclei were extracted with the zwitterionic detergent empigen to create an enriched source of the higher molecular weight nucleoporins. Empigen purified NPC were mixed with PICs from macrophages infected with wild type or NLS mutant viruses, incubated for 2 hours at 4 degrees in the presence of protease inhibitors and mild RIPA buffer, then immunoprecipitated with anti-integrase N-terminus antibody mAb6G5 and protein A sepharose. Immunoprecipitated complexes were washed with buffer, heat denatured, run on 4-20% Tris-Glycine gel, then blotted onto nitrocellulose. The blot was then probed with mAb414, a monoclonal antibody that is specific for FG-containing nucleoporins. **A)** Western blot of immunoprecipitated PICs/nucleoporins. Several diffuse smaller bands of unknown identity are visible in the WT, D116A, and K236/240A wells. **B)** Western blot of empigen extracted rat liver NPC probed with mAb414 [ref 83]. **C)** Schematic diagram of the nucleoporins as they appear in the NPC [ref 122]. Data shown are representative of three independent experiments.

Construction of Plasmids

EGFP-IN expression vectors contained the coding sequence for enhanced green fluorescent protein (Clontech), upstream of an HIV-1 IN open reading frame derived from the CXCR4-tropic, HXB-2 subclone, R73. This gene fusion was cloned into pcDNA3.1 (+)-CTE, a derivative of pcDNA3.1 (+) (Invitrogen), that includes the constitutive transport element (CTE) from Mason-Pfizer monkey virus. A synthetic, codon-modified IN gene, IN-MOD (GenBank Accession #AF422697), was constructed by primer extension of overlapping 100 base-pair oligo-nucleotide segments and cloned into pcDNA3.1 (+)-CTE to create the modified integrase expression plasmid, pcDNA3.1 (+) IN-MOD-CTE. The natural version of the viral integrase gene from R73, IN-VIR, was cloned into pcDNA3.1 (+)-CTE to create the cognate, viral integrase expression vector, pcDNA3.1 (+) IN-VIR-CTE. These plasmids are identical except for the segment encoding the wild-type integrase amino acid sequence. For the plasmids used in the eGFP-MBP-IN NLS transfection and nucleofection studies, the C-terminal portion of the IN MOD gene was amplified by PCR and inserted at the C-terminal end of a gene encoding an enhanced green fluorescent protein (eGFP)-maltose binding protein (MBP) fusion protein in the context of a CMV expression vector plasmid.

For viral studies, the relevant mutations were subcloned into derivatives of R73. In some cases, the envelope gene of R73 was replaced with that of the macrophage-tropic primary isolate, R73(YU-2).

Preparation of Primary Immune cell populations

Immature Dendritic Cells

Primary human immature dendritic cells were prepared by CD14+ magnetic bead separation of CD14+ monocytes from peripheral blood mononuclear cells (PBMCs) obtained from blood donors. In general, 50ml of a human leukocyte concentrate (from 500ml of whole blood, LEUKOPAK (from the New York Blood Center) is mixed 1:1 with 1X phosphate buffered saline (PBS) in a T75 culture flask. Approximately 33ml of this 1:1 PBS:Blood mixture is layered onto 10ml of Ficoll paque (Pharmacia) into each of three 50ml Falcon polypropylene tubes, then tubes are centrifuged at 2000 rpm for 20 minutes at room temperature with the brake off. Following this centrifugation step, a layer of PBMCs is visable in each tube between the plasma (top) and red blood cells (bottom). This layer of PBMCs is collected from each tube and placed in a fresh 50ml Falcon tube then the volume is adjusted to 50ml with PBS. Cells are then centrifuged at 1200 rpm for 10 minutes at 4°C to pellet the PBMCs. The PBMC pellet is then resuspended

in 3ml of ACK red blood cell lysis buffer (Bio-Whittaker) and incubated at room temperature for 5 minutes. The volume is then adjusted to 50ml with cold PBS and the cells are centrifuged at 1200 rpm for 10 minutes at 4°C to pellet. Cells are then resuspended and washed with 50ml cold PBS two more times. A 10µl aliquot was taken before the final centrifugation, added to 10µl of trypan blue solution (Cellgro) on a piece of parafilm, then added to a hemocytomer for cell counting while the tube of PBMCs is spinning.

The PBMC pellet was then resuspended in MACS buffer (0.5% Bovine Serum Albumin/PBS) by adding 80µl of MACS buffer per 1 x 10^7 PBMCs. CD14+ magnetic beads (Miltenyi Biotech) were added at 10µl per 1 x 10^7 cells, mixed by pipetting and stored at 4°C for 20 minutes to allow for binding. Cells were then washed by adding 20-fold increase of volume of MACS buffer, then centrifugation at 1200 rpm. CD14+ labeled PBMCs were then resuspended in 1ml of cold MACS buffer, and added to a washed (with 5ml of cold MACS buffer) LS magnetic column mounted on a mini-MACS magnetic stand (Miltenyi Biotech). Cells were allowed to flow through by gravity then the column was washed twice with 5ml of MACS buffer. The CD14- fraction (flow through) was collected and saved for the preparation of CD4+ T cells (see below). The CD14+ monocytes were collected by removing the LS column from the magnet, adding 5ml of MACS buffer, then pushing the cells through with a plunger into a 15ml

Falcon tube. Aliquots (10µl) were taken for counting, and cells were centrifuged at 1200 rpm for 10 minutes at 4°C.

CD14+ monocytes were resuspended at a concentration of 1 x 10^6 cells/ml in cell culture medium, supplemented with cytokines. The cell culture medium used throughout was complete RPMI 1640 (Cellgro) supplemented with 2mM L-glutamine (GIBCO-BRL Life Technologies), 50µM 2-mercaptoethanol (Sigma), 10mM HEPES (GIBCO-BRL Life Technologies), penicillin (100 U/ml)-streptomycin (100µg/ml) (GIBCO-BRL Life Technologies), and 1% heparinized human plasma (R1 media). Recombinant human (rh) IL-4, was purchased from R&D Systems and used at a final concentration of 100 Units/ml , recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) was bought from Immunex and used at a final concentration of 1000U/ml. Every two days, 200µl of fresh R1 media was added to each well supplemented with GM-CSF (1000 U/ml) and IL-4 (100 U/ml). Immature dendritic cells were then harvested between days 6-8, and a small aliquot was removed for FACs analysis.

CD4+ T cells

Primary human CD4+ T cells were isolated by negative selection using the CD14-PBL fractions from the immature DC preps described above. Briefly, a cocktail of antibody-conjugated magnetic beads (Miltenyi Biotech) was added to CD14-

PBLs which include: anti-CD8 (removes CD8+ CTLs), anti-HLA-DR (removes residual APCs), anti-CD11b (removes monocytes and granulocytes), and anti-CD19 (removes B cells). The magnetic beads were added at an equal fraction based on the parameters described above (10μl of each type per 10^7 CD14- cells). Cells were incubated with beads for 20 minutes at 4°C, then washed by adding 20-fold increase of volume of MACS buffer, then centrifugation at 1200 rpm. CD4+ T cells were negatively selected by adding them to and collecting the flow through from a LS column attached to a miniMACS magnet as described above. CD4+ T cells were then pelleted by centrifugation at 1200rpm for 10 minutes. After centrifugation, cells were resuspended at high density (1×10^7 cells/ml) in R1 media supplemented with 5ng/ml of recombinant human IL-2 (Boehringer Mannheim) and maintained in culture with fresh media added every 2 days until use.

Macrophages

Primary human macrophages were derived from the same CD14+ monocyte populations used to make immature DC above. Briefly, CD14+ monocytes were resuspended in R1 media and plated at 1×10^5 cells per cm^2 in 24-well tissue culture plates containing round 12 mm glass slides (Fisher Scientific) that had been treated beforehand with poly-L-lysine (Sigma), for 20 minutes at room temperature, then washed twice with PBS. Monocytes were incubated in a 37°C

incubator with 5% CO_2 for six to seven days, then the R1 media was removed and replaced with fresh Dulbecco's Modified Eagle Medium (DMEM, Cellgro), supplemented with 10% Fetal Bovine Serum (FBS, Cellgro).

FACS analysis of Primary Immune cells

Phenotyping of immature dendritic cells

For FACs analysis of primary human immature dendritic cells, $2x10^4$ DCs per well of a 96-well V-bottomed plate (100µl per well). Fluorescently-labeled antibodies were added at the following concentrations per 100µl cells: IgG1PE(1:50), IgG2bPE (1:50), IgG2a-FITC(1:200) controls, IgG1PE (1:50), IgG2bPE (1:50), HLA-DR-FITC (1:200), CD25-PE(1:50)/HLA-DR-FITC(1:200), CD80-PE(1:50)/HLA-DR-FITC, CD83-PE (1:50) (Immunotech)/HLA-DR-FITC, and CD86-PE (1:50)/HLA-DR-FITC. FITC and PE-conjugated antibodies were from BD Pharmingen unless otherwise noted. The V-bottomed plate was then covered with aluminum foil and incubated for 20 minutes (or overnight) at 4°C in the dark in a refrigerator. After incubating with antibodies, 100µl of FACS wash (PBS/5% FCS/0.1% Azide) was added, and mixed by pipetting. Then the cells in each well were pelleted by centrifuging the plate for 2-3 minutes at ~2500rpm. Aspirated off approximately half of the volume, added 100µl of FACS wash, centrifuge at the above time and speed and repeat twice more (4 spins in total After the 4^{th} spin, aspirate of half of the volume and add 100µl of FACS fix (10%

formalin in PBS). Store the fixed, stained cells in the fridge (covered in foil) until ready to analyze on FACs Calibur fluorescence-activated cell sorter (Becton Dickinson). The cells were acquired (5-10,000 events), gating on large DCs and the HLA-DR$^+$ cells were assessed. Immature DCs are HLA-DR$^+$ and express moderate CD86 and at best low levels of CD80, while CD25 and CD83 are typically not expressed on the immature DC surface (CD83 is expressed intracellularly). All of the DC used in the nucleofection and other experiments described in this thesis were of the immature phenotype.

Phenotyping of CD4+ T cells

FACs analysis of CD4+ T cells was performed in the same manner as for the immature dendritic cells described above, with the exception that different antibodies were used for staining. Briefly, CD4+ T cells were stained with the same controls: IgG1PE(1:50), IgG2bPE (1:50), IgG2a-FITC(1:200), IgG1PE (1:50), IgG2bPE (1:50), and the following specific antibodies, along with a constant CD3-FITC-labeled antibody: CD4-PE(1:50)/CD3-FITC(1:50), CD8-PE(1:50)/CD3-FITC, CD11b-PE(1:50)/CD3-FITC, CD14-PE(1:50)/CD3-FITC, CD25-PE(1:50)/CD3-FITC, CD38-APC(CalTag)(1:50)/CD3-FITC, and CD69-PE(1:20)/FITC. FACS analysis was performed as above on the FACs Calibur sorter gating on small CD3+ T cells (5-10,000 events). The CD4+ cells used in the nucleofection experiments were CD4-high, CD8-

negative, CD14-negative, CD25-low, CD38-low, and CD69-low, indicating a naïve CD4+ T cell phenotype.

Cell Culture, Transfections, Nucleofections, and Virus Production

HEK293T and CEM-SS cells were maintained in the appropriate media (DMEM (Cellgro) supplemented with 10% Fetal Bovine Serum (Cellgro). Macrophages were prepared by monocyte isolation from the peripheral blood mononuclear cells (PBMC) of normal blood donors either by density gradient centrifugation on 46% Percoll (Amersham Pharmacia Biotech), followed by plastic adherence for 1 hour at 37°C and culture for 6 days in RPMI-1640 with 2% Human AB serum (Sigma) or by CD14-magnetic bead cell sorting (MACS procedure, Miltenyi Biotech, described above) before infection. For primary dendritic cells and CD4+ T cells, ficoll purified PBMCs were incubated with CD14-specific microbeads (Miltenyi Biotech) and CD14+ monocytes were isolated over LS columns according to the manufacturer's protocol. These CD14+ monocytes were cultured in GM-CSF (100 U/ml) and IL-4 (10 U/ml) for 6 days, with fresh cytokines and media added every two days until cells resembled immature dendritic cells on FACS analysis as described above. Activated CD8-depleted peripheral blood leukocytes (PBL) were generated from the same donor-matched

PBMC samples used to prepare the macrophages, by removing CD8 positive cells from the CD14- fractions remaining after monocyte removal with anti-CD8 magnetic beads according to the manufacturer's protocol (Dynal, Inc.). All viral stocks were prepared in transfected 293T cells filtered through 0.45 mm filters and treated with DNase I (Sigma) at 8 mg/ml for 30 minutes at 37°C.

Nucleofection with AMAXA kit

For nucleofection experiments, the remaining, CD14- cells were subjected to further purification using magnetic beads specific for HLA-DR, CD11b, CD19, and CD8 and a population of approximately 96% (by FACS) pure CD4+ T cells was isolated and cultured at high density (1 x 10^7 per ml) in media supplemented with 5ng/ml recombinant human IL-2. At day six of each culture, cells were collected, pelleted by centrifugation, and resuspended to 2.5 x 10^5 cells/ml in either T cell nucleofection solution (AMAXA Biotech) or dendritic cell nucleofection solution depending on the cell type. One hundred microliters of either cell suspension was mixed with 5μg of each eGFP-MBP-IN plasmid DNA in a microfuge tube, then transferred to a specialized electroporation cuvette and placed in the nucleofection device using program U-14 or U-02 according to the manufacturer's protocol (AMAXA Biotech). Nucleofected cells were immediately re-equilabrated in warm (37°C) media and incubated at 37°C with

5% CO_2 for 12 hours. After 12 hours, cell aliquots from each condition were taken and adhered (in serum free media, 100µl cells/slide) to alcian blue/poly-L-lysine (Sigma) coated 12 mm circular glass slides (Fisher Scientific), then fixed with 4% paraformaldehyde (Tusoumis), permeabilized with 0.1% Trition-X100 / PBS (Sigma), washed 3 times with PBS, and mounted with DAPI containing mounting medium (Vectashield). In some experiments, nucleofected CD4+ T cells were sorted for GFP expression (Peter Lopez, ADARC Mo-Flo facility) and a more concentrated population of nucleofected T cells were used to make slides for analysis on DeltaVision. This resulted in clearer microscope images, as much of the post-nucleofection dead cell debris was removed by the sorting.

Generation of HIV Virus and Lentiviral Vector Stocks

All virus and lentiviral vector stocks were generated by transient transfection for plasmid DNAs into HEK 293T cells. Briefly, 4 x 10^6 cells were seeded into 75 cm^2 tissue culture flasks (Falcon BD). After 12-16 hours, a transfection mixture containing viral DNA (10µg of each HIV R73 plasmid DNA), the cationic lipid DMRIE-C (30µl) (Invitrogen), and Opti-MEM media (6ml) (Invitrogen) was added to the cells and allowed to incubate on cells for 16-24 hours. Transfection mixture was then removed and fresh DMEM-10% FBS media was added back to the transfected cells. Cell supernatants containing virions were harvested at 24 -48 hours later, filtered through 0.45µm syringe filter (Millipore), and stored

frozen in 1ml aliquots at -80°C until use. In the case of lentiviral vector stocks, 10μg of an expression plasmid, 15μg of a packaging plasmid, and 5μg of a VSV-g envelope plasmid, and 50μl DMRIE-C were co-transfected under the same conditions as above. For generating the Vpr-GFP HIV virus stocks, 10μg of a Vpr- HIV-R73-Yu2 molecular clone plasmid DNA was added with 5μg of a plasmid specifying a Vpr-GFP fusion protein (kind gift of Dr. Tom Hope, University of Chicago), along with 30μl of DMRIE-C reagent in 6ml total volume of Opti-MEM, and transfection of 293T cells was performed as described above. For the still images shown in Figure 22, 500ng p24 was added to each well of macrophages in a 24-well plate (at an approximate MOI 10,000).

Real-Time PCR Assays

The PCR primers used to amplify and detect the various viral DNA intermediates are described below and their coordinates within the R73 plasmid used in these experiments is indicated.

Early reverse transcription assay--forward primer:

5'-TCTCTGGCTAACTAGGGAACCCACTGCTT-3'(bp181-209 in R region of LTR) , 491-519 in R73

reverse primer: 5′-TGACTAAAAGGGTCTGAGGGATCTCTAGTTACCAG-3′, (bp271-302 in U5 region of LTR) 581-613 in R73 molecular beacon: 56-FAM-5′-CCGAACCAGTAGTGTGTGCCCGTCTGTTGTGTGGTTCGG-3′-Dabcyl (bp243 -266 in U5 region of LTR) 552-576 in R73

Late reverse transcription assay--forward primer: 5′-AGATCCCTCAGACCCTTTTAGTCAGTGTGG-3′ (592-621), reverse primer: 5′-GCCGCCCCTCGCCTCTTG-3′, (721-738) beacon: 56-FAM-5′-CCGACCCTCTCGACGCAGGACTCGGCTTGGGTCGG-3′-Dabcyl. 684-705

2-LTR circle assay--forward primer: 5′-CTCAGACCCTTTTAGTCAGTGTGGAAAATCTCTA-3′, reverse primer: 5′-TGACCCCTGGCCCTGGTGTGTAG-3′, beacon: 56-FAM-5′-CCGCACCTACCACACACAAGGCTACTTCGTGCGG-3′-Dabcyl. 53-74 in R73

CCR5 assay--forward primer: 5′-TCATTACACCTGCAGCTCTCATTTTCCATACAGTC-3′, reverse primer: 5′-CACCGAAGCAGAGTTTTTAGGATTCCCGAGTA-3′, beacon: 56-FAM-5′-GCGCCTATGACAAGCAGCGGCAGGAGGCGC-3′-Dabcyl.

Contamination control –forward primer:

5'- TACTCTAAACCTGTGATTCCTC-3' 119-140 in R73 plasmid "backbone"

reverse primer: 5'-TGACCCCTGGCCCTGGTGTGTAG-3', 289-311

beacon: 56-FAM-5'-CCGCACCTACCACACACAAGGCTACTTCGTGCGG-3'-Dabcyl.

For each reaction, five microliters (approximately 100ng) of total genomic DNA was added to 45μL of PCR mastermix containing 1X PCR buffer II (Perkin Elmer), 1X ROX internal passive reference buffer, 200nM dNTPs, 400 nM primers, 200nM molecular beacon, and 1 U AmpliTaq Gold (Perkin Elmer) in each well of a 96-well PCR reaction plate. After an initial denaturation step (94°C for 10 minutes), PCR amplification proceeded for 50 cycles (94°C for 15 sec; 60°C for 30 sec; 72°C for 30 sec) in a Perkin Elmer ABI Prism 7700 sequence detection system. Copy numbers for all samples were determined by extrapolation from a plot of PCR standards using the ABI Prism 7700 SDS quantification software.

Immunocytochemistry and Basic Fluorescence Microscopy

Cells were grown on poly-L-lysine (Sigma)-treated glass coverslips. After transfection, cells were fixed with 3% electron-microscopy grade para-formaldehyde (Tosoumis) in PBS, and, in some cases, stained using mouse

monoclonal antibodies to IN (generally mAb6G5, NIH AIDS reagent repository), and either a tyramide-based amplification with anti-HRP antibodies (TSA kit, AppliedBiosystems) or, in the case of Nup358 staining, a biotinylated anti-rabbit antibody conjugated to a streptavidin-linked quantum dot 655 (qDots Inc). Coverslips were mounted in medium containing DAPI (Vectashield, Vector Laboratories). EGFP, DAPI and antibody fluorescence were visualized using deconvolution, restoration microscopy (DeltaVision, Applied Precision). In the case of nucleofected cells, dendritic cells and T cells were resuspended in serum-free RPMI (100µl) and adhered to alcian blue coated glass coverslips for 5 minutes at 37°C, then 2 minutes at 4°C, fix with 3% paraformaldehyde, and inverted onto glass slides with DAPI containing mounting medium (Vectashield).

Viral Infections

Macrophage and PBL infections

Virus was added to confluent a monolayer of macrophage (approximately 2.0 x 10^5 cells) or activated PBLs (1.0 x 10^6) in 24-well format and infection enhanced by spinoculation. Briefly, the technique of spinoculation involves spinning the virus particles (in media containing Fetal Bovine Serum) down onto the monolayer of macrophages or other cells by centrifugation for 90 minutes at 2300

rpm at room temperature. This technique increases the amount of virus that contacts the cell surface and enhances the rate of infection in the cultures. Generally cells were infected with between 250-50ng p24 of HIV viruses or lentiviral vector stocks, with no large differences apparent in downstream assays (i.e. Real Time PCR) when the spinoculation technique was used. Following spinoculation, infected cells were washed thoroughly (at least 3 times) with PBS to remove free virions and excess contaminating viral DNA from the transfected stocks. Fresh media was added back to all washed infected cells. Inhibitors used include 10mM UC78154 (in PBS), 5mM L-731,988 (in DMSO) . Total nucleic acid was obtained using the QIAmp Blood Kit according to the manufacturer's protocol (Qiagen).

Digitonin-permeabilized macrophage nuclear import assays

Primary human macrophages were grown on glass coverslips in 24-well tissue culture plates. Cells were washed twice with ice-cold 1X transport buffer (20mM HEPES, 110mM potassium acetate, 2mM magnesium acetate, and 0.5mM EGTA) with 1X protease inhibitors (EDTA-free mini complete tablets, Roche), and then permeabilized for 5 minutes on ice in transport buffer containing 45μg/ml high-purity digitonin (Calbiochem). After one wash in transport buffer, slides (with permeabilized macrophages) were removed from the wells of the tissue culture

plate and inverted onto 30µl nuclear import reactions containing 10µl cell extract (from Xenopus oocytes or HeLa cells), 1µl of 20X stock of an energy-regenerating system (1mM ATP, 1mM GTP, 2mM creatine phosphate, 40 U/ml creatine phosphokinase), 1µl of 10mM GTP, 1µM of recombinant protein of interest (or 10µl PICs), and 1µl of recombinant Ran. Nuclear import reactions were incubated at 37°C for 2 hours, then cells were washed twice with 1X transport buffer, once in PBS, fixed with 3% PFA and immunostained and visualized as described above.

PIC Immunoprecipitations

To generate PIC from infected macrophage cytosol, three million monocytes were plated in T25 flasks (Falcon), incubated at 37°C for six days in R1 media, and media was changed to DMEM/10%FBS on day 7. In a Biosafety Level 2+ facility and inside of a tissue culture hood, primary human macrophages were infected with 3µg R73-Yu2 virus stock in 3ml culture media, and incubated for 12 hours, to allow reverse transcription to occur. Then, virus was washed off of cells with PBS (a total of 4 washes, 6ml volume per wash), and cells were incubated in 3ml hypotonic lysis buffer A (10mM HEPES, 1.5mM MgCl2, 5mM KCl, 5mM DTT, 1X protease inhibitors, and 50µg/ml digitonin (CalbioChem)) for 1 minute, then

buffer was removed, and 1ml of hypotonic lysis buffer A was added back to the infected cells. The flask was then incubated on ice for 30 minutes, then lysed cells were removed from the flask with a cell scraper, and the 1ml of infected cell lysate was collected in a 1.5ml microfuge tube. A small piece of parafilm was placed over the mouth of the tube to reduce aerosol formation. The infected cell lysate was then homogenized by passing it through a blunt-ended 23 gauge needle attached to a 1 cc syringe for 5-10 minutes (about 50-100 times). The homogenized lysate was then centrifuged at 6000 rpm for 5 minutes at 4°C to pellet the cell nuclei. The supernatant was removed, placed in a new tube, and centrifuged at 10,000 rpm for 10 minutes at 4°C. The clarified supernatant ("PICs") was then collected and stored in 100µl aliquots in a -80°C freezer until use.

Empigen-extracted rat liver nucleoporins were made by detergent solubilization of rat liver nuclear envelopes obtained from Elias Coutavas in the Blobel lab (prepared as previously described by Blobel and Potter, 1966). Briefly, three hundred million nuclei were thawed and pelleted at 3000 rpm for 1 minute. Pelleted nuclei were resuspended with constant vortexing at a final concentration of 100 U/ml 1 by drop-wise addition of buffer A (0.1mM $MgCl_2$, 1mM DTT, 0.5mM PMSF, 1 $\mu g/ml^{-1}$ leupeptin/pepstatin/aprotinin) supplemented with 5$\mu g/ml^{-1}$ DNase I and 5$\mu g/ml^{-1}$ RNase A. After resuspension, nuclei were

immediately diluted to 20 U/ml^{-1} by addition of buffer B (buffer A 10% sucrose, 20mM triethanolamine, pH 8.5) with constant vortexing. After digestion at room temperature for 15 minutes, the suspension was underlayed with 4ml ice-cold buffer C (buffer A 30% sucrose, 20mM triethanolamine, pH 7.5) and centrifuged at 3,500 g for 10 minutes in a swinging bucket rotor (Sorvall SH-3000). The pellet was resuspended in ice-cold buffer D (buffer A 10% sucrose, 20mM triethanolamine, pH 7.5) at a final concentration of 100 U/ml^{-1}. The suspension was diluted to 67 U/ml^{-1} with buffer D 0.3 mg/ml^{-1} heparin (Sigma), and then immediately underlayed and pelleted as above. The heparin pellet was resuspended in ice-cold buffer D (100 U/ ml^{-1}), diluted to 67 U/ ml^{-1} with buffer D+3% Triton X-100 (Sigma), and 0.075% SDS, then pelleted as above. The pellet (the NPC-lamina fraction) was then resuspended in buffer D+0.3% Empigen (CalbioChem) (final concentration of 100 U/ml^{-1}). After incubation on ice for 10 minutes, the insoluble lamina was separated from soluble nucleoporins by centrifugation in a microfuge at 12,000 rpm for 15 minutes. The supernatant, containing the Empigen-extracted rat liver nucleoporins was then collected, snap frozen in liquid nitrogen, and stored in 100µl aliquots at -80°C until use.

For the immunoprecipitations, 100µl of thawed PICs was added to 100µl of Empigen-extracted rat liver nuclei. This mixture was then used "pre-cleared" with Protein A/G sepharose beads (Santa Cruz Biotech) by adding 20µl of beads

per 200µl of sample, incubating at 4°C on a rotator, then centrigufing at 14,000rpm for 1 minute. Monoclonal antibodies to the N-terminus of HIV integrase (1:100 dilution of mAb-6G5, NIH AIDS repository) were then added to each sample, along with 20µl of PBS-washed Protein A/G beads. Immunoprecipitation reactions were incubated at 4°C for 3 hours on a rotator, then beads were harvested by centrifugation at 14,000 rpm for 1 minute. The beads were washed six times with 400µl of IP buffer (1X Tris-buffered saline (TBS) + 1X protease inhibitors + 0.1mM $MgCl_2$), then 50µl of 2X Laemmli sample buffer (BioRad, supplemented with 1mM DTT) was added to each sample, then tubes were incubated at 95°C for 5 minutes on a heat block. Samples were then electrophoresed on 4-20% Tris-Glycine gels (Invitrogen), transferred onto nitrocellulose (Amersham), and analysed by standard western blotting procedures (1X Tris-Glycine transfer buffer, with 10% methanol and 0.1% SDS, Invitrogen, then transferred for 2 hours at a constant 30V) using the anti-FG nucleoporin monoclonal antibody mAb-414 from the Blobel lab (1:2000 dilution).

Ligand blot assays

For the ligand blot assays, 1 x 10^6 rat liver nuclei were run out in each well of a 15-well 4-20% Tris-Glycine gel, then transferred to nitrocellulose by western blotting as above. The blot was then blocked for 1 hour with 3% non-fat dry milk

(BioRad) and then cut by well into 15 strips. Each strip was air-dried, then added face up to each well of an 8-well plate (Costar). Using both wild type and NLS mutant version of recombinant HIV integrase (kindly provided by Dr. Daria Hazuda, Merck & Co., Inc.) at a final concentration of 1μM per well, 1ml of recombinant protein solution was added to each strip. The strips were then washed six times with 2ml of IP buffer, and incubated for 1 hour at room temperature with 1ml of a 1:500 dilution of mAb-6G5 also in IP buffer. The strips were then washed again, and incubated for 30 minutes with a goat-anti-mouse-horseradish peroxidase-conjugated secondary antibody (Pharmacia) at a 1:40,000 dilution. Bound HIV integrase was then detected by developing the strips with the Super-signal West-Pico HRP substrate reagent (Pierce), according to the manufacturer's protocol.

Live-Cell Imaging with Vpr-GFP viruses

Primary human macrophages were grown in special Petri dishes with embedded glass slides (Matek, Inc.). Prior to addition of Vpr-virus stocks (made in HEK 293T cells as above, by co-transfecting viral constructs lacking the Vpr gene with plasmids encoding Vpr-GFP), the Petri dish of macrophages was mounted onto an Axioplan microscope (Zeiss) equipped with a spinning-disk laser confocal set up and a heated chamber (37°C). D116A and K236/240A NLS mutant viruses

were added (3μg p24 of each virus, 3ml total volume, approximate MOI of 10,000) and focus was adjusted until optimal fields of virions and cells were attained. Once an optimal field was identified, the microscope software was programmed to image the cells over a defined duration (16 hours) at 5-minute intervals through a range of Z series positions through the cell. At the end of the 16-hour infection period, images from the middle section of the Z series (representing the center of the cell with relation to the top and bottom of the culture dish) were compiled into live-motion movies using the imaging software (MetaMorph 6.0, Universal Imaging Systems) showing virus dynamics inside of live cells.

Chapter 7. Appendix and glossary of terms and Abbreviations

AIDS Acquired Immune Deficiency Syndrome

BAF Barrier to Autiointegration Factor

CA Capsid

CMV Cytomegalovirus

cPPT Central Polypurine Tract

EGFP Enhanced Green Fluorescent Protein

56FAM 56-carboxyfluorescein

GST Glutathione S-Transferase

HIV-1 Human Immunodeficiency Virus type 1

HMG High Mobility Group Protein

IN Integrase

Kap α Karyopherin α

Kap β Karyopherin β

LTR Long Terminal Repeat

MA Matrix

MBP Maltose-Binding Protein

MLV Moloney Leukemia Virus

NC Nucleocapsid

NFAT-1 Nuclear Factor of Activated T-cells -1

NLS Nuclear Localization Signal

NPC Nuclear Pore Complex

Nup Nucleoporin

PBL Peripheral Blood Lymphocytes

PBS Phosphate Buffered Saline

PCR Polymerase Chain Reaction

PIC Pre-Integration Complex

PFA Paraformaldehyde

PR Protease

RRE Rev-Responsive Element

RT Reverse Transcriptase

SIV Simian Immunodeficiency Virus

TAR Tat-Activated Region

WGA Wheat Germ Agglutinin

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