Université de Montréal

# Integrase, the Central DNA Flap and Human Immunodeficiency Virus Type 1 Nuclear Import

Par
Zhu-jun Ao
Programme de Biologie Moléculaire
Faculté des études supérieures




Thèse présentée à la Faculté des études supérieures
en vue de l'obtention du grade de
Philosophie Doctoral (Ph.D)
en Biologie Moléculaire


October, 2006

Université de Montrèal
Faculté des études supérieures

Cette thèse intitulée:

# Integrase, the Central DNA Flap and Human Immunodeficiency Virus Type 1 Nuclear Import

Présenté par:
Zhu-Jun Ao

A été évaluée par un jury composé des personnes suivantes

| | |
|---|---|
| Dr. Hugo Soudeyns: | Président-rapporteur |
| Dr. Xiaojian Yao: | Directeur de recherche |
| Dr. Eric A Cohen: | Co- Directeur de recherche |
| Dr. Ali Ahmad: | Membre du jury |
| Dr. Chen Liang | Examinateur externe |

Thèse acceptée le:..... October, 2006

**IN MEMORY OF MY MOTHER**

# SUMMARY

One of the hallmarks that distinguishes Human Immunodeficiency Virus Type 1 (HIV-1) from other oncoretroviruses is its capacity to productively infect nondividing cells. At the molecular level, this ability of HIV-1 to infect non-dividing cells was attributed to the karyophilic properties of the HIV-1 preintegration complex (PIC). How HIV-1 PIC successfully enters the nucleus and what viral and cellular factors are involved in this process have been the focus of many recent studies. Here, we studied the functional roles of two HIV-1 elements, the central DNA flap and integrase (IN), in the nuclear import by: 1) investigating the impact of the central DNA flap on HIV-1 nuclear import and viral replication; 2) analyzing the contribution of different regions in the C-terminal domain of HIV-1 IN to it nuclear localization as well as their effects on virus infection; 3) detecting the interaction of HIV-1 IN with importin 7 (Imp7) and analyzing its functional role in IN nuclear accumulation.

A factor proposed to regulate HIV-1 nuclear import is the special structure presented in the reverse transcribed viral DNA, called the central DNA flap. It is a region of triple-stranded DNA created by two discrete half-genomic fragments with a central strand displacement event controlled *in cis* by a central polypurine tract (cPPT) and a central termination sequence (CTS) located in the 3'- region of IN gene during HIV-1 reverse transcription. The exact impact of the central DNA flap on the early steps of HIV-1 infection is still an open question. In this study, we took advantage of the ability of Vpr-RT-IN fusion proteins to trans-complement RT and IN defects of HIV-1 viruses, and delineated RT and IN gene sequences that are important for HIV-1 replication in single-round replication system. Our results revealed that the presence of central DNA flap element confers a 5- to 8-fold infectious advantage to single-cycle replicating virus in a variety of cellular systems. We further investigated the impact of the central DNA flap on HIV-1 reverse transcription, nuclear import and integration by PCR

analysis. The results indicated that the central DNA flap enhances the establishment of HIV-1 infection in single-round replication assays primarily by facilitating nuclear import of proviral DNA.

HIV-1 IN plays a key role in viral cDNA integration into the host chromosome. In addition to its role for viral DNA integration, it has also been shown to assist other critical steps of early stage HIV-1 replication, including reverse transcription and nuclear import of viral DNA. IN has been well documented to possess karyophilic properties. By using mutagenic analysis, we defined that the tri-lysine regions $^{235}$WKGPAKLLWKGEGAVV and $^{211}$KELQKQITK in the C-terminal domain of HIV-1 IN contributed to its karyophilic property, while mutations at the arginine/lysine rich region ($^{262}$RRKAK) had no significant effect. Analysis of their effects on viral infection in a VSV-G pseudotyped RT/IN trans-complemented HIV-1 single cycle replication system revealed that all three C-terminal mutant viruses (KK215,9AA, KK240,4AE and RK263,4AA) exhibited more severe defect of infectivity than IN class I mutant D64E in HeLa-CD4-CCR5-β-Gal cells, and in dividing as well as nondividing C8166 T cells. By analyzing viral cDNA synthesis, nuclear import, and integration, we found that the two tri-lysine regions, especially the region KK215,9AA, significantly impaired nuclear import step, whereas all C-terminal mutants inhibited viral reverse transcription to different extents. Taken together, these results indicated that the C-terminal domain of HIV-1 IN plays an important role for both HIV-1 reverse transcription and that viral DNA nuclear import and different regions in this domain may be preferentially involved in each of these early steps during HIV-1 replication.

Next, we investigated the potential mechanism(s) involved in the action of HIV-1 IN during HIV-1 nuclear import. We demonstrated that HIV-1 IN, but not MAp17, specifically interacts with cellular nuclear import receptor Imp7 in the cells. Genetic analysis revealed that the C-terminal domain of IN is the region responsible for interaction between IN with Imp7, and an IN mutant

(KK240,4AA/RK263,4AA) disrupted the Imp7-binding ability of the protein. Using a VSV-G pseudotyped HIV single-cycle replication system, we demonstrated that the IN/imp7 interaction deficient mutant completely inhibited the replication of HIV-1 and displayed impairment at both viral reverse transcription and nuclear import steps. Moreover, transient knockdown of Imp7 in both HIV-1 producing and target cells resulted in 2.5 to 3.5-fold inhibition of HIV infection. Altogether, our results indicate that HIV-1 IN specifically interacts with Imp7 and this viral/cellular protein interaction contributes to an efficient HIV-1 infection.

Altogether, our studies indicate that the HIV-1 central DNA flap and IN are two important factors for HIV nuclear import in both dividing and nondividing cells. Moreover, we demonstrated that the two tri-lysine regions in the C-terminal domain of HIV-1 IN play an important role for viral DNA nuclear import, but the $^{262}$RRKAK region appears preferentially involved in reverse transcription and perhaps integration. In order to understand the mechanism involved in IN action during early viral replication, we found that HIV-1 IN specifically interacts with cellular nuclear import receptor Imp7 *in vivo* and this viral/cellular protein interaction contributes to an efficient HIV-1 infection. Both regions ($^{235}$WKGPAKLLWKG and $^{262}$RRKAK) within the C-terminal domain of IN are important for efficient IN/Imp7 interaction. Further studies are required to determine the exact functional role of IN-Imp7 interaction and to identify other cellular importing that may be necessary and/or coordinate with Imp7 to contribute to HIV-1 IN action during HIV-1 nuclear import and replication.

Key words: Integrase, the central DNA flap, HIV-1 PIC, Nuclear Import, Importin 7

# RÉSUMÉ

Une des caractéristiques qui différencient le virus de l'immunodéficience humaine de type 1 (VIH-1) des autres oncorétrovirus est sa capacité à infecter des cellules non-mitotiques de façon productive. Au niveau moléculaire, cette habileté du VIH-1 a été attribuée aux propriétés karyophiles de son complexe de préintégration (CPI). L'étude du mécanisme permettant au CPI du VIH-1 de pénétrer dans le noyau et l'identification des facteurs viraux et cellulaires impliqués dans ce processus ont fait l'objet de plusieurs recherches récentes. J'ai analysé dans l'étude présente les rôles fonctionnels de deux éléments du VIH-1, le flap central de l'ADN et l'intégrase (IN) dans l'import nucléaire des 3 façons suivantes : 1) en étudiant l'effet du flap central de l'ADN sur l'import nucléaire du VIH-1 et la réplication virale; 2) en analysant la contribution des différentes régions du domaine C-terminal de l'IN du VIH-1 sur la localisation nucléaire de la protéine ainsi que leurs effets sur l'infection virale; 3) en étudiant l'interaction de l'IN avec importine 7 (Imp7) et en analysant son rôle fonctionnel dans l'accumulation de IN dans le noyau.

Un des facteurs proposés pour la régulation de la translocation nucléaire du VIH-1 est une structure particulière présente dans l'ADN viral rétrotranscrit, le flap central de l'ADN. Le flap central est une région d'ADN à trois brins créée par deux fragments discrets semi-génomiques avec un événement de déplacement de brin central contrôlé en cis par la séquence centrale de polypurine (cPPT) et une séquence de terminaison centrale localisée dans la région 3' du gène IN durant la transcription inverse du VIH. L'impact précis du flap central de l'ADN sur les étapes précoces de l'infection par le VIH-1 est une question qui demeure controversée. Dans cette étude, nous avons pris avantage de la capacité des protéines de fusion Vpr-RT-IN à trans-complémenter les défectuosités de la RT et de l'IN virales et nous avons identifié les séquences des gènes de la RT et de l'IN

qui sont importantes pour la réplication du VIH-1 dans un système de réplication à cycle unique. Nos résultats révèlent que la présence de l'élément flap central de l'ADN confère un avantage au niveau du pouvoir infectant, qui est de 5 à 8 fois plus élevé pour un virus se répliquant pendant un seul cycle dans de multiples systèmes cellulaires. De plus, nous avons étudié l'impact du flap central de l'ADN sur la transcription inverse du VIH-1, l'import nucléaire et l'intégration par une analyse de'PCR. Les résultats indiquent que le flap central de l'ADN aide av développement d'une infection par le VIH-1 dans des tests de réplication à cycle unique, principalement en facilitant l'import nucléaire de l'ADN proviral.

L'IN du VIH-1 joue un rôle primordial dans l'intégration de l'ADNc viral dans le chromosome hôte. En plus de son rôle dans l'intégration virale, il a été démontré que l'IN est impliquée dans d'autres étapes cruciales au début de la réplication du VIH-1, incluant la transcription inverse et l'import nucléaire de l'ADN viral. Il est bien documenté que l'IN possède des propriétés karyophiles. Par une analyse de mutagenèse, nous avons déterminé que les régions triple-lysine $^{235}$WKGPAKLLWKGEGAVV et $^{211}$KELQKQITK du domaine C-terminal de l'IN du VIH-1 contribuent à son caractère karyophile, tandis qu'une mutation au niveau de la région riche en arginine/leucine ($^{262}$RRKAK) n'a aucun effet significatif. L'analyse de leur effet sur l'infection virale dans un pseudotype du VSV-G exprimant RT/IN et qui trans-complémente le système de réplication à cycle unique du VIH-1 révèle que les trois virus ayant des mutations au C-terminus (KK215,9AA, KK240,4AE et RK263,4AA) ont une défectuosité plus importante au niveau du pouvoir infectant qu'un mutant d'IN de classe 1 D64E dans des cellules HeLa-CD4-CCR5-β-Gal, et que dans des cellules T C8166 mitotiques et non-mitotiques. En analysant la synthèse de l'ADNc viral, l'import nucléaire et l'intégration, nous avons déterminé que les deux régions tri-lysine, et surtout la région KK215, 9AA, ont un effet détrimental significatif sur l'import nucléaire, tandis que tous les mutants C-terminaux ont inhibé la transcription

inverse virale à des degrés variables. Ces résultats mis ensemble indiquent que le domaine C-terminal de l'IN du VIH-1 joue un rôle important et pour la transcription inverse et pour l'import nucléaire de l'ADNc du VIH-1. De plus, différentes régions de ce domaine pourraient préférentiellement être impliquées dans chacune de ces étapes précoces de la réplication du VIH-1.

Nous avons ensuite tenté de déterminer le(s) mécanisme(s) potentiel(s) impliqué(s) dans le rôle joué par l'IN du VIH-1 durant l'import nucléaire du VIH-1. Nous avons démontré que l'IN du VIH-1, et non Map17, interagit spécifiquement avec le récepteur de l'import nucléaire Importine 7 (Imp 7) dans les cellules hôtes. Étonnamment, le domaine de l'IN responsable de l'interaction avec Imp7 est localisé dans le C-terminus de la protéine, un mutant IN (KK240,4AA/RK263,4AA) affecte négativement son habileté de liaison à Imp7 de façon significative. L'analyse de l'effet de ce mutant sur l'infection virale dans un pseudotype de VSV-G qui trans-complémente le système de réplication à cycle unique du VIH-1 révèle un inhibition complet de la réplication virale et un effet détrimental sur la transcription inverse virale et l'import nucléaire. De plus, l'inactivation transitoire d'Imp7 dans les cellules productrices ou cibles réduit l'infection avec le VIH de 2.5-3.5 fois. Ces résultats mis ensemble indiquent que l'interaction entre la protéine virale IN du VIH et la protéine cellulaire Imp7 est important pour l'efficacité de l'infection avec le VIH.

Ces études indiquent que le flap central de l'ADN et l'IN du VIH-1 sont deux facteurs importants pour l'import nucléaire du VIH dans les cellules mitotiques et non-mitotiques. De plus, nous avons démontré que les deux régions de tri-lysine du domaine C-terminal de l'IN du VIH-1 jouent un rôle important dans l'import nucléaire de l'ADN viral, mais que la région $^{262}$RRKAK est préférentiellement impliquée dans la transcription inverse et peut-être aussi dans l'intégration. Quant au mécanisme d'action de l'IN durant la réplication virale

précoce, nous avons déterminé que l'IN du VIH-1 interagit spécifiquement avec le récepteur de l'import nucléaire cellulaire Imp7 *in vivo* et l'interaction entre ces protéines virales et cellulaiers est important pour l'efficacité de l'infection ave le VIH. Deux régions ($^{235}$WKGPAKLLWKG et $^{262}$RRKAK ) dans le domaine C-terminal de l'IN sont important pour un interaction efficace entre IN et Imp7. D'autres études sont nécessaires pour déterminer le rôle fonctionnel précis de l'interaction IN-Imp7 et d'identifier d'autres importines cellulaires qui pourraient être requises avec ou sans Imp7 pour l'action de l'IN du VIH-1 pendant l'import nucléaire et la réplication du VIH-1.

Mots : Intégrase, le flap central de l'ADN, VIH-1 CPI, import nucléaire, Importine 7.

# ACKNOWLEDGEMENTS

I would like to take this opportunity to express my deepest gratitude and appreciation to my supervisors Dr. Xiaojian Yao and Dr. Eric A. Cohen. I thank them for providing me with an opportunity to enter a wonderful world of HIV research from histology where I had ever worked for many years. I thank them for their continuous guidance and helpful suggests through the five-year program and for their patience, generosity and inspiration. It is difficult for me to put into words the impact that they have had on me both professionally and personally. I will never forget the years I lived and worked in Montreal and Winnipeg.

I would like to thank the Department of Microbiology and Immunology and all members of the Department for the continued support and for always challenging me to develop into a better scientist. I am grateful to my PhD committee members for timely suggestions and encouragement during the past 5 years.

Many thanks to all those involved in this project in one way or another over the past years, in particular: Dr. Keith R Fowke and Dr. Alan W. Cochrane, Guanyou Huang, Zaikun Xu, Han Yao and Meaghan Labine,

Thanks to all my Lab-mates in Montreal and Winnipeg, for their love and support, in particular, I want to thank Andres Finizi, John Rutherford, Nicole Rougeau and Johanner Mercier for their technical support. I would also like to thank Mélanie Welman, Yong Xiao and Julie Binette for the valued information and support they provided.

Special thanks and appreciation to my family, especially my son for walking me through these years with great understanding, patience and love.

# PREFACE

This PhD thesis was written according to the Guide Concerning Thesis Preparation from the Faculté des etudes supérieures at Université de Montréal. The structure and contents of the thesis conform to the options, subject to the approval of their department, of including, as part of their thesis, copy of the text of a paper submitted for publication, or original texts of published papers. The thesis includes, as separate chapters or sections: 1) a table of contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rational and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the study, when the review is appropriate, and (5) a general discussion and future direction.

I have included, as chapters of this thesis, the original texts of two published papers, and one original manuscript, which have been submitted for publication. The papers presented in the thesis are the following:

1.Zhujun Ao, Xiaojian Yao and Éric A. Cohen. 2004. Assessment of the Role of the Central DNA Flap in Human Immunodeficiency Virus Type 1 Replication using a Single-Cycle Replication System. J. Virol.78: 3170-3177

2.Zhujun Ao, Keith R. Fowke, Éric A. Cohen, Xiaojian Yao. 2005. Contribution of the C-terminal tri-lysine regions of Human Immunodeficiency Virus Type 1 integrase for efficient reverse transcription and viral DNA nuclear import. Retrovirology. 2:62

3. Zhujun Ao*, Guanyou Huang*, Zaikun Xu, Han Yao, Meaghan Labine, Alan W. Cochrane, and Xiaojian Yao. 2006. Interaction of Human Immunodeficiency Virus Type 1 integrase with the cellular nuclear import receptor importin 7 and its impact on viral replication. Submitted to J. Biol. Chem. (* both authors contributed equally to this work).

# TABLE OF CONTENTS

# LIST OF FIGURES

**Chapter III**



**Chapter IV**

# LIST OF ABBREVIATIONS

| | |
|---|---|
| APOBEC3G | :APOBEC3G (A Poliprotein B mRNA-editing Enzyme-Catalytic Polypeptide-like 3G) |
| AD | :Adenoviruses |
| AIDS | :Acquired Immune Deficiency Syndrome |
| BAF | :Barrier-to-Autointegration Factor |
| CA | :Capsid |
| CCD | :Catalytic Core Domain |
| CPPT | :Central copy of the Polypurine tract cis-active sequence |
| CTD | :C-terminal Domain |
| CTS | :Central Termination Sequence |
| CypA | :Cyclophilin A |
| Env | :Envelope glycoprotein |
| FG | :Phenylalanine-glycine repeats |
| Gag | :Group-specific antigen |
| H1 | :Histone 1 |
| HAART | :Highly Active Anti-Retroviral Therapy |
| HBV | :Hepatitis B virus |
| HEAT | :Named after the proteins in which similar sequences were first identified: Huntingtin, Elongation factor 3, the A subunit of protein phosphatase 2A, and the Tor1 kinase) |
| HIV-1 | :Human Immunodeficiency Virus Type 1 |
| HMGI (Y) | :High Mobility Group Protein Y |
| HSV | :Herpes Simplex Virus |
| IBB | :Importin-β Binding domain |
| IN | :Integrase |
| INI-1 | :Integrase Interactor 1 |
| INIs | :Integrase Inhibitors |
| IP | :Immunoprecipitation |
| Kap | :Karyopherin |
| LAPO-2α | :Lamina-associated polypeptide |
| LEDGF/p75 | :The human lens epithelium-derived growth actor/transcription coactivator p75 |
| LEM | :Named for LAP2, Emerin, and MAN1 |
| MA | :Matrix |
| MAGI | :The Multinuclear Activation of Galactosidase Indicator Assay |
| MHC I | :Major Histocompatibility Class I |
| MLV | :Murine Leukemia Virus |
| MVBs | :Multivesicular Bodies |
| MM-PCR | :Mu-mediated PCR |

| | |
|---|---|
| NC | :Nucleocapsid |
| Nef | :Negative factor |
| NF-κB | :Nuclear Transcription factor κB |
| NLSs | :Nuclear Localization Signals |
| NPC | :Nuclear Pore Complex |
| NTD | :N-terminal domain |
| Nups | :Nucleoporins |
| PIC | :Preintegration Complex |
| Pol | :Polymerase protein |
| PR | :Protease |
| PTHrP | :Parathyroid homone-related protein |
| RanGEF | :Ran Guanine nucleotide exchange factor |
| RanGAP | :Ran GTPase activating protein |
| RRE | :Rev Responsive Element |
| RQ-PCR | :Real-time Quantitative Polymerase Chain Reaction |
| RT | :Reverse transcriptase |
| RTCs | :Reverse-transcription complex |
| SV40 | :Simian virus 40 |
| TSG101 | :Human Tumor Susceptibility Gene 101 |
| Vif | :Viral infectivity factor |
| Vpr | :Viral protein R |
| Vpu | :Viral protein U |
| YFP | :Yellow- fluorescent protein |

# Chapter I

# Literature review

HIV-1 is the cause of acquired immune deficiency syndrome, AIDS. Twenty years following its discovery, the virus remains a major threat to public health and a challenge for drug development. Currently HIV-1 infects an estimated 40 million individuals worldwide. The present therapeutic drug strategies such as highly active anti-retroviral therapy (HAART) that target the viral enzymes reverse transcriptase (RT) and protease (PR) have been successful in slowing disease progression and reducing the incidence of AIDS and AIDS-related mortality. However, these therapies, while suppressing HIV-1 replication to undetectable levels, still cannot eradicate the infection. Therefore, the anti-HIV treatment needs to be continued throughout the patient's life. Due to the occurrence of toxicity, metabolic disorders and the emergence of drug resistant HIV strains, alternative treatment strategies are urgently required.

Indeed, extensive efforts have been made for the development of effective therapeutic agents to block the function of another HIV-1 enzymatic molecule, integrase (IN), and/or to target other HIV-1 replication steps, including HIV-1 nuclear translocation–a process critical for HIV replication. Moreover, much research has also been focused on the definition of specific interactions between HIV-1 and cellular proteins and trying to open an opportunity to block HIV replication.

In this chapter, a mini-review of HIV-1 replication cycle will be provided. Afterwards, the discussion will be focused primarily on the research progress on HIV-1 nuclear import including the roles of the DNA flap and IN, and also on the IN's structure, its multiple functions as well as its cellular co-factors. Finally, a brief review of HIV-1 IN inhibitors (INIs) will be provided.

## I.1 HIV-1 GENOME AND REPLICATION CYCLE

### I.1.1 HIV-1 genome and viral proteins:

HIV-1 belongs to family retroviridae, genus lentivirus. The mature HIV virion is an enveloped, roughly spherical particle with a diameter of 110nm. The

genome consists of two identical linear positive-sense single stranded RNA molecules enclosed by a conical capsid composed of viral protein, p24. The viral RT, IN, nucleocapsid (NC) protein and also some regulatory and accessory viral proteins are enclosed in the capsid (Fig.I.1.A).

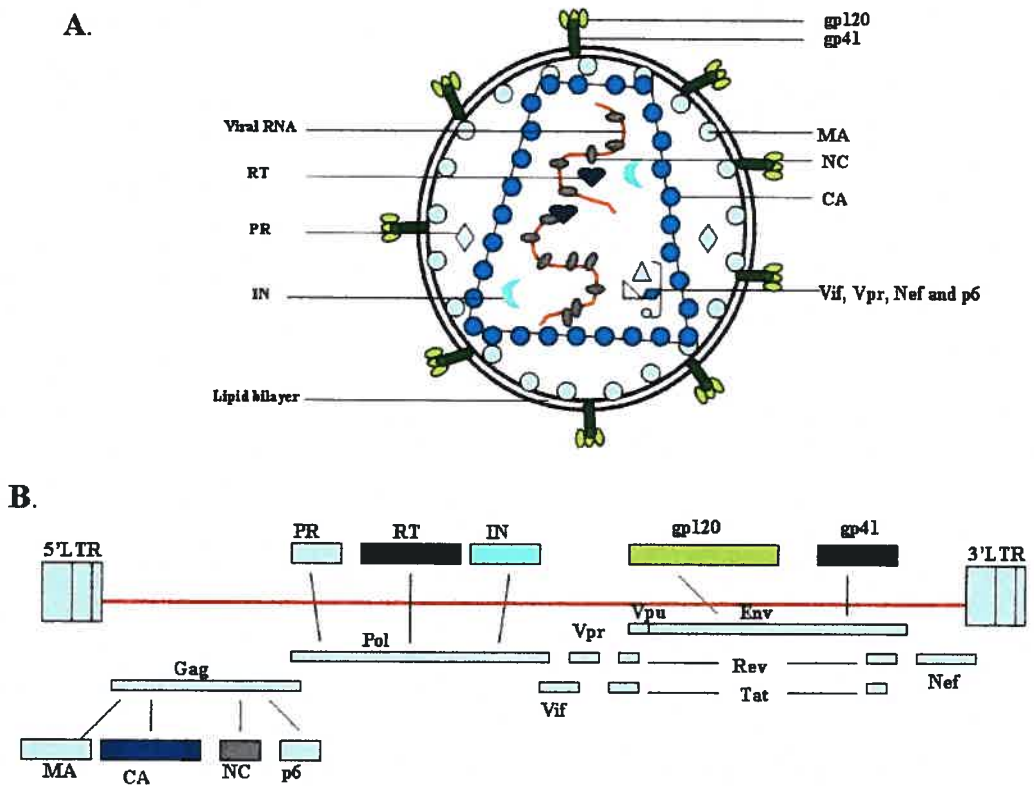


**Fig.I.1. HIV-1 genome and virion structure.** A. Schematic representation of mature HIV-1 particle. Position of the proteins and genomic RNA are indicated. B. Organization of the HIV-1 genome and its structure and enzymatic gene products.

The HIV-1 genome is encoded by a 9-kb RNA that encodes structural and non-structural proteins (Fig.I.1.B). The structural genes include *gag* (group-specific antigen), *pol* (polymerase), and *env* (envelope glycoprotein). The *gag* gene encodes a polyprotein precursor, Pr55 $^{Gag}$, that is cleaved by the viral protease (PR) to the mature Gag proteins matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p7), and p6. Two spacer peptides, p2 and p1, are also

generated upon $Pr55^{Gag}$ processing. The *pol*-encoded enzymes are initially synthesized as part of a large polyprotein precursor, $Pr160^{GagPol}$, whose synthesis results from a rare frame shifting event during $Pr55^{Gag}$ translation. The individual *pol*-encoded enzymes, PR, RT, and IN, are cleaved from $Pr160^{GagPol}$ by the viral PR. The envelope glycoprotein is also synthesized as a polyprotein precursor, gp160, which is processed by a cellular protease into the surface (SU) Env glycoprotein gp120 and the transmembrane (TM) glycoprotein gp41. The Gag proteins and Env proteins make up the core of the virion and outer membrane envelop, respectively, and pol proteins provide essential enzymatic functions and are also encapsulated within the particle. Two genomic-length RNA molecules are packaged in the particle (Fig.I.1).

In addition to the *gag, pol* and *env* genes, HIV-1 also encodes a number of regulatory and accessory proteins: Tat is critical for transcription from the HIV-1 LTR and Rev plays a major role in the transport of viral RNAs from the nucleus to the cytoplasm. Vpu, Vif, Vpr and Nef have been termed "accessory" or "auxiliary" proteins to reflect the fact that they are not uniformly required for virus replication *in vitro*. However, *in vivo*, these proteins contribute, to varying degrees, to efficient virus replication and disease induction.

Vpu (Viral protein U) is an 81 amino acid integral membrane phosphoprotein that is unique to HIV-1 (Cohen, Terwilliger et al. 1988; Strebel, Klimkait et al. 1988). It performs two major functions during HIV-1 replication: 1) it enhances the release of virus particles (Terwilliger, Cohen et al. 1989), and 2) promotes the degradation of CD4 through the host ubiquitin/proteasome pathway (Margottin, Bour et al. 1998).

Vpr (viral protein R), a 14-kDa, 96 amino acid protein, is incorporated efficiently into virions by a specific interaction with the p6 gag protein (Cohen, Dehni et al. 1990; Paxton, Connor et al. 1993; Kondo, Mammano et al. 1995; Yao, Kobinger et al. 1999). Vpr can rapidly and efficiently arrest cell in G2 phase, induce apoptosis, weakly stimulate gene expression from the HIV LTR and

might play a role in nuclear import of the viral preintegration complex (PIC) (Heinzinger, Bukinsky et al. 1994; Connor, Chen et al. 1995; Nie, Bergeron et al. 1998; Vodicka, Koepp et al. 1998).

Vif (viral infectivity factor) is a basic protein of 23kDa which is packaged into virions and is required in virus-producing cells during the late stages of infection to enhance viral infectivity by 10-to-1000 fold (Strebel, Daugherty et al. 1987; Kao, Akari et al. 2003). Vif mutation can cause profound defects in virus infectivity. The defective phenotype is cell-type dependent and is determined by the virus-producing cell. Thus, certain cell lines (for example HeLa, COS, 293T, SupT1, CEM-SS and Jurkat) are "permissive" for Vif mutants; virus produced from these cell lines is fully infectious regardless of the target cell used. In contrast, other cell types (macrophages, primary human T cells and some restrictive T cell line) are "non-permissive" (Borman, Quillent et al. 1995; Madani and Kabat 2000). This cell-type specificity suggests that host factors play a role in Vif function. In 2002, Sheehy et al. found that non-permissive cells contain an anti-viral cellular factor termed APOBEC3G (A Poliprotein B mRNA-editing Enzyme-Catalytic Polypeptide-like 3G), and that the anti-viral action of APOBEC3G is thwarted by Vif (Sheehy, Gaddis et al. 2002). The current mechanism proposed for protection of the virus by HIV-1 Vif is to induce APOBEC3G degradation through an ubiquitination-dependent proteasomal pathway, thereby occluding its incorporation into the virion (Liu, Yu et al. 2004).

Nef (negative factor) is a 27kDa, membrane-associated phosphoprotein. Its membrane binding is dependent upon a myristic acid moiety covalently attached to the N-terminus of the protein. Nef has been detected at low levels in viral particles, where it localizes to the virion core. Several primary Nef functions have been reported: 1) down regulation of CD4 and major histocompatibility class I (MHC I) molecules from the cell surface; 2) stimulation of virus infectivity; 3) modulation of cellular activation pathway.

**I.1.2 HIV-1 replication** (Fig.I.2): The life cycle of HIV proceeds through a series of events that can be divided into two distinct phases: "early" and "late". The early phase refers to the steps from cell binding to the integration of viral DNA into the host chromosome, whereas the late phase begins with the expression of viral genes and goes through the release and maturation of viral particles. Along this journey, HIV-1 hijacks the cellular machinery, while at the same time counteracting cellular defenses.

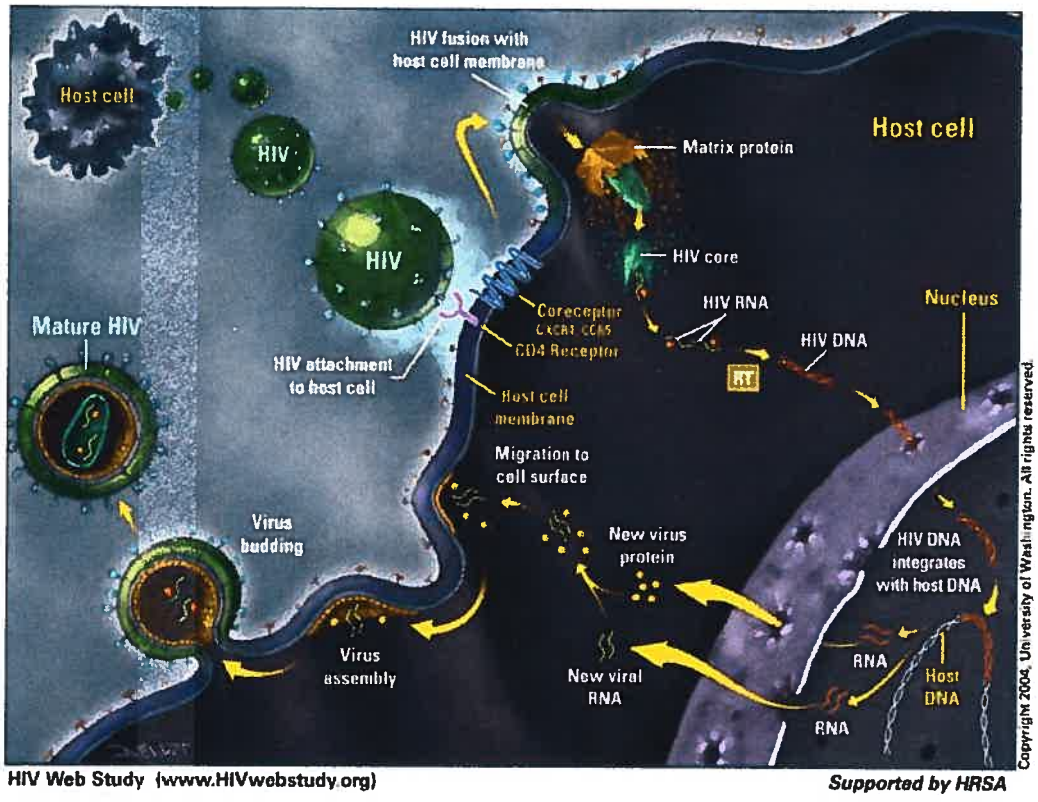


**Fig.I.2. Schematic representation of the HIV-1 life cycle.** The major steps in the early and late stages of the replication cycle are indicated (www.HIVwebstudy.org, Copyright 2004, University of Washington. All rights reserved)

**1). Virus Entry:** The entry process consists of receptor binding followed by coreceptor binding, and ultimately membrane fusion allowing the viral core to enter the cell. First, the viral gp120 binds specifically to CD4. This causes

conformational changes in gp120, which then exposes coreceptor binding sites. Depending on the viral tropism, determined by the V3 loop of gp120, cellular co-receptors CCR5 or CXCR4 are engaged. It is at this point when fusion pore formation begins (Markosyan, Cohen et al. 2003). The gp41 ectodomain adopts a hypothetical extended conformation; the fusion peptide at the N-terminus of gp41 inserts directly into the target cell lipid bilayer. The N- and C-helices of the gp41 ectodomain fold into a highly stable six-helix bundle, bringing the membranes in apposition and allowing membrane fusion to occur and subsequently deliver HIV core to the cytoplasm (Melikyan, Markosyan et al. 2000).

**2). Uncoating and reverse transcription**: The events that follow viral penetration into host cells known as uncoating, require the viral core to undergo a partial and progressive disassembly in order to release viral gene and leads to the generation of reverse-transcription complexes (RTCs). The uncoating process of HIV-1 is poorly understood. It is possible that the penetration process itself may trigger the uncoating process. However, specific cellular or viral factors may also play a role during this event (Dvorin and Malim 2003).

It is suggested that initiation of reverse transcription is coupled to the onset of uncoating of the viral core (Zhang, Dornadula et al. 2000). The viral RNA genome is reverse transcribed by the virion-packaged RT enzyme, generating a linear double-stranded DNA molecule (Fig.I.9). The fidelity of the reverse transcription is influenced by the presence of cellular protein APOBEC3G (Goncalves, Korin et al. 1996; Mangeat, Turelli et al. 2003; Mariani, Chen et al. 2003; Zhang, Yang et al. 2003). HIV-1 Vif counteracts the antiretroviral effect of the cellular protein APOBEC3G by reducing its expression and incorporation into progeny virions (Sheehy, Gaddis et al. 2002; Kao, Khan et al. 2003; Mariani, Chen et al. 2003). Since viral protein Nef enhances viral DNA synthesis, it has been proposed to act either at the level of viral uncoating or reverse transcription only when it occurs by fusion at the plasma membrane (Aiken and Trono 1995;

Aiken 1997). In addition, the host protein cyclophilin A (CypA) was found to enhance HIV infectivity during early post-entry events by counteracting the inhibitory activity of human host restriction factor Ref1 and allowing reverse transcription to be completed (Towers, Hatziioannou et al. 2003).

In the case of retroviruses, after penetration into the host cells, viruses have to reach their sites of replication, the nucleus. Research has shown that HIV-1 cores use microtubules to transport its genome toward the cell nucleus (McDonald, Vodicka et al. 2002). It is during this time that the RTC and PIC are formed. The precise composition of HIV-1 RTCs and PICs is still a matter of debate. It should be noted that PICs are usually defined as the preintegration competent complexes, whereas reverse transcription is incomplete in RTCs. Most studies show that PICs contain viral DNA, viral proteins PR, RT, IN, Vpr, NC and host proteins such as the high mobility group protein HMGI (Y) and the human lens epithelium-derived growth actor/transcription coactivator p75 (LEDGF/p75) (Farnet and Bushman 1997; Llano, Vanegas et al. 2004).

**3). Nuclear import:** The HIV-1 cDNA, associated to viral and cellular proteins, has to cross the nuclear membrane to reach the host chromatin for its integration. The mechanism by which the PIC translocates into the nucleus remains to be defined. It is recognized that HIV-1 PIC enters the nucleoplasm through the nuclear pore complex (NPC) by active transport (Weinberg 1991; Bukrinsky, I.N. et al. 1992). The current model is that the HIV proteins present in PICs harbors karyophilic properties, either directly by bearing nuclear localization signal (NLS) or indirectly by interacting with karyophilic cellular proteins. The viral proteins MA, Vpr and IN, and a DNA structure, the central DNA flap, have been implicated in the HIV-1 nuclear import (Bukrinsky, Haggerty et al. 1993; Heinzinger, Bukinsky et al. 1994; Gallay, Hope et al. 1997; Nie, Bergeron et al. 1998; Vodicka, Koepp et al. 1998; Haffar, Popov et al. 2000; Zennou, Petit et al. 2000; de Noronha, Sherman et al. 2001). Besides the role of viral proteins in

HIV-1 nuclear import, further studies are clearly required to elucidate additional cellular proteins involved in this step. HIV-1 nuclear import will be discussed in much greater detail in part I.3.

**4). Integration:** The viral-encoded enzyme IN will catalyze the insertion of the linear, double-stranded viral DNA into the host cell chromosome. Although the process of proviral integration has been intensively studied with *in vitro* assays, the molecular basis of *in vivo* integration and the selection of integration sites remain poorly understood. Recent studies have given a global picture of integration preferences of retroviruses such as HIV-1 and murine leukemia virus (MLV), revealing that proviral integration of both retroviruses preferentially occurs in genes highly transcribed by RNA Pol II (Schroder, Shinn et al. 2002; Wu and Marsh 2003). Interestingly, HIV-1 proviruses are found on the entire length of the transcriptional unit, whereas MLV integration events distribute evenly upstream and downstream of the transcriptional start site of actively transcribed genes, ±1kb from the CpG islands. The regional preferences of HIV-1 along the host genome, in the absence of sequence specificity, strongly suggest the existence of tethering mechanisms between components of the PIC and cellular partners, directing the PIC to its final destination (Bushman 2002; Bushman 2003). In fact, Ciuffi et al, found that the cellular protein LEDGF/p75 affects the choice of target sites for HIV-1 integration (Ciuffi, Llano et al. 2005). The HIV-1 integration reaction will be discussed in much greater detail in part I.4.

**5). Gene Expression** (Fig I.3)**:** Following nuclear import of the viral PIC and prior to integration, the viral DNA can be found in the nucleus in three forms: linear DNA, 1-LTR, or 2-LTR circles (Farnet and Haseltine 1991; Bukrinsky, I.N. et al. 1992; Wu 2004). Nef, Tat and Rev are produced in basal amounts from these DNA forms through activation of LTR promoter by cellular factors such as nuclear transcription factor κB (NF-κB) (Aiken and Trono 1995; Wu 2004).

Transcription of the HIV provirus is characterized by an early, Tat-independent phase and a late, Tat-dependent phase. In the absence of the viral transactivator Tat, a series of short transcripts are produced due to inefficient elongation by the recruited RNA pol II and the HIV promoter is strictly under the control of the local chromatin environment and cellular transcritption. This process results in the synthesis of basal amounts of Tat protein (Kao, Calman et al. 1987; Jordan, Defechereux et al. 2001). RNA synthesis is greatly increased when Tat is present. Tat activates transcription through binding to TAR element of LTR and to other transcriptional activators of cellular origin (Harrich, Ulich et al. 1996; Harrich and Hooker 2002). Transcription from the HIV-1 LTR leads to the generation of a large number of viral RNA that fall into three major classes, unspliced RNAs, single-spliced mRNAs and multiply spliced mRNA (Fisher, Feinberg et al. 1986). The unspliced and partially spliced mRNAs are transported to the cytoplasm by viral protein Rev which binds to a structure called RRE (Rev responsive element) present in unspliced or partially spliced RNA (Pollard and Malim 1998). The single-spliced *env* gene is used for translation of the precursor protein gp160, which is glycosylated within the endoplasmic reticulum. The unspliced RNAs are used for translation of Gag and Gag-Pol polyproteins and also serve as viral genomic RNA for progeny. Then, the assembly process starts.

**6). Viral assembly:** The major player in virus assembly is the Gag precursor polyprotein, $Pr55^{Gag}$ (Freed 1998). The HIV-1 uncleaved Gag polyprotein contains three domains that play very important roles in the assembly and budding processes. These domains are referred to as the membrane targeting (M), interaction (I) and late (L) domains. The M domain is located within the MA region, which is myristylated on its N-terminal glycine thereby targeting Gag to the plasma membrane. Once Gag has arrived at the plasma membrane, it must engage in Gag-Gag (as well as Gag-lipid and Gag-RNA) interactions to enable the assembly of progeny virions to take place. The I domain is responsible for Gag

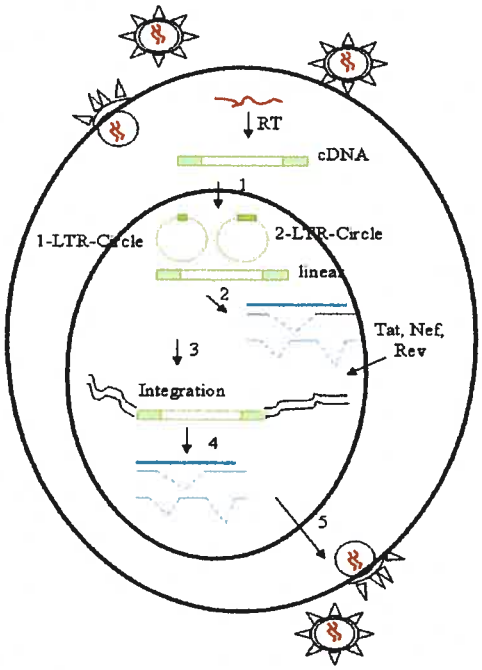


**Figure I.3. The model of transcription from pre-integrated viral DNA and provirus.** Following nuclear import (step1) and prior to integration, the non-integrated DNA, in the forms of linear, 1-LTR- or 2-LTR-circles, is active in transcribing all three classes of viral transcripts (step2). The multiply spliced, early transcripts such as *tat, nef* and *rev* are also translated into products. The non-spliced and singly spliced viral transcripts encoding viral structural proteins are not translated. Following viral integration (step3), post-integration transcription initiates (step4). Expression of these transcripts leads to production of progeny virions (step5).

monomer interaction and is located within the C-terminus of CA, the p2 spacer peptide, and the N-terminal portion of NC (Freed 1998; Sandefur, Smith et al. 2000; Derdowski, Ding et al. 2004). After translation, the Env protein migrates and inserts into the plasma membrane. Gag and Gag-Pol also move to the cellular membrane and start to assemble directed by the Gag polyprotein (Sandefur, Smith et al. 2000; Barreca, Lee et al. 2003; Derdowski, Ding et al. 2004). In addition, full-size genomic RNA is packaged into the immature core. The specific encapsidation of retroviral RNAs into virus particles is mediated by interactions between the packaging signal in RNA and the NC domain of Gag (Berkowitz,

Ohagen et al. 1995; Damgaard, Dyhr-Mikkelsen et al. 1998; De Guzman, Wu et al. 1998; Kleiman and Cen 2004). So MA forms the inner shell of the particle, located just under the viral membrane. CA forms the conical capsid that encloses the viral genomic RNA. NC interacts with the viral RNA within the capsid. During virus assembly, CypA is also packaged into HIV virions by binding to the N-terminal domain of CA and was found to enhance HIV infectivity during early post-entry events (Luban, Bossolt et al. 1993; Franke, Yuan et al. 1994; Braaten, Aberham et al. 1996).

7). **Budding:** Once the virion is assembled successfully, the viral membrane must separate (bud) from the cellular membrane. Retroviral budding is mediated by the L-domain located in the p6 region of Gag (Wills and Craven 1991; Huang, Orenstein et al. 1995). Deletion of p6, or mutations within a highly conserved Pro-Thr-Ala-Pro (PTAP) motif located near the N-terminus of p6, resulted in a marked effect in budding where viruses are unable to pinch off from the plasma membrane (Gottlinger, Dorfman et al. 1991; Huang, Orenstein et al. 1995). Additionally, the L domain requires modification by mono-ubiquitylation to mediate budding (Vogt 2000; Alroy, Tuvia et al. 2005). The identification of PTAP motif led to studies that identified a cellular protein, TSG101, that interacts with this L domain. Tsg101 and the vacuolar protein-sorting pathway are essential for HIV-1 budding (Garrus, von Schwedler et al. 2001). The realization that the vesicular sorting system mediates HIV-1 budding has led to the suggestion that budding can also take place in MVBs (Multivesicular bodies), which clearly appears to be the case in macrophages (Orenstein, Meltzer et al. 1988; Mellman and Steinman 2001; Raposo, Moore et al. 2002; Thery, Zitvogel et al. 2002). Thus, there are now two major models for HIV-1 budding. One is the lipid raft budding model (Nguyen and Hildreth 2000). The other model is the Trojan exosome hypothesis (Gould, Booth et al. 2003), which is largely based on the evidence that HIV-1 assembles in the MVB and buds into this compartment in

primary macrophages. Nevertheless, advances in the past several years have revealed that key components of the cellular endosomal sorting machinery are critical for budding. The information on the PTAP-TSG101 interaction site provides the impetus for studies aimed at developing small-molecule inhibitors that block the Gag-TSG101 interaction and thereby impair virus budding.

**8). Maturation**: During or shortly after virus release from the plasma membrane, the viral PR cleaves the Gag and Gag-Pol polyprotein precursors to generate the mature Gag and Pol proteins (Fig.I.1.B). The most visible outcome of HIV-1 maturation is that virion morphology is converted from containing an electron-lucent center to containing an electron-dense, conical core. The failure of the virion to mature properly is associated with a complete loss of infectivity. Studies showed that extensive regions of *Pol* are required for efficient HIV polyprotein processing and particle maturation (Ross, Fuerst et al. 1991; Quillent, Borman et al. 1996). HIV-1 Vif protein has been shown to inhibit the proteolytic activity of HIV-1 protease *in vitro* and in bacteria, a function assigned to its N-terminal domain (Kotler, Simm et al. 1997; Baraz, Friedler et al. 1998; Potash, Bentsman et al. 1998; Friedler, Blumenzweig et al. 1999).

## I.2 NUCLEAR TRANSPORT IN THE CELL

### I.2.1. Gateway to the nucleus: The nuclear pore complex

The only means for proteins and RNA-protein complexes (RNPs) to move between the nucleus and cytoplasm in order to perform their normal cell functions is through the nuclear pore complex (NPC). NPCs are huge, symmetric structures present in the double membrane of the nuclear envelope that has a mass of 60MDa and composed of relatively few (30) proteins termed nucleoporins (Nups) in vertebrates (Cronshaw, Krutchinsky et al. 2002). Electron microscopy (EM) has revealed the elaborate structure of NPC, which are consisting of at least three separate structural elements: (I) Cytoplasm fibers, (II) the central core, and (III)

the nuclear basket (Fig.I.4). There is a single channel through which all transport proceeds. The diameter of the channel appears to be flexible, and can expand from 10nm to 25nm to translocate large cargos.

There are three groups of Nups: 1) those that are composed of integral membrane proteins and are believed to play a role in NPC assembly and anchoring of NPC to the membrane; 2) structural Nups; and 3) those that contain phenylalanine-glycine (FG) repeats. The FG-repeat Nups make up about half the mass of the NPC and most are distributed symmetrically, although a few are found only on the nuclear or cytoplasmic face. It has been shown that karyopherins (Kap) can associate directly with FG repeats (Ryan, McCaffery et al. 2003), and that there are as many as 200 FG-repeat Nups per NPC, representing a large number of Kap-binding sites (Rout, Aitchison et al. 2003). A study revealed that adenovirus 2 binds directly to the CAN/Nup214 nucleoporin (Trotman, Mosberger et al. 2001). Recently, by using Nup98-depleted NPC by the interfering RNA (siRNA) technique, Ebina et al, showed that Nup98 on the NPC specifically participates in the nuclear entry of HIV-1 cDNA (Ebina, Aoki et al. 2004).

### 1.2.2 Import to the nucleus

The central pore of the NPC only permits free passive diffusion of molecules smaller than 9 nm in diameter. In order for large molecules to enter the nucleus at a physiologically useful rate, they must be actively transported by using kap and other soluble factor, including the small GTPase Ran (Macara 2001; Fried and Kutay 2003; Weis 2003). A broad spectrums of macromolecules cross the nuclear envelope, including mRNAs, tRNAs, ribosomal proteins, ribosomal subunits, snRNP and many soluble proteins and also viral gene of many nuclear-replicating viruses. Although viruses can wait in the cytosol until the cell undergoes mitosis, this restricts infection to dividing cells and so is of limited use for most viruses. Therefore, some viruses have developed strategies

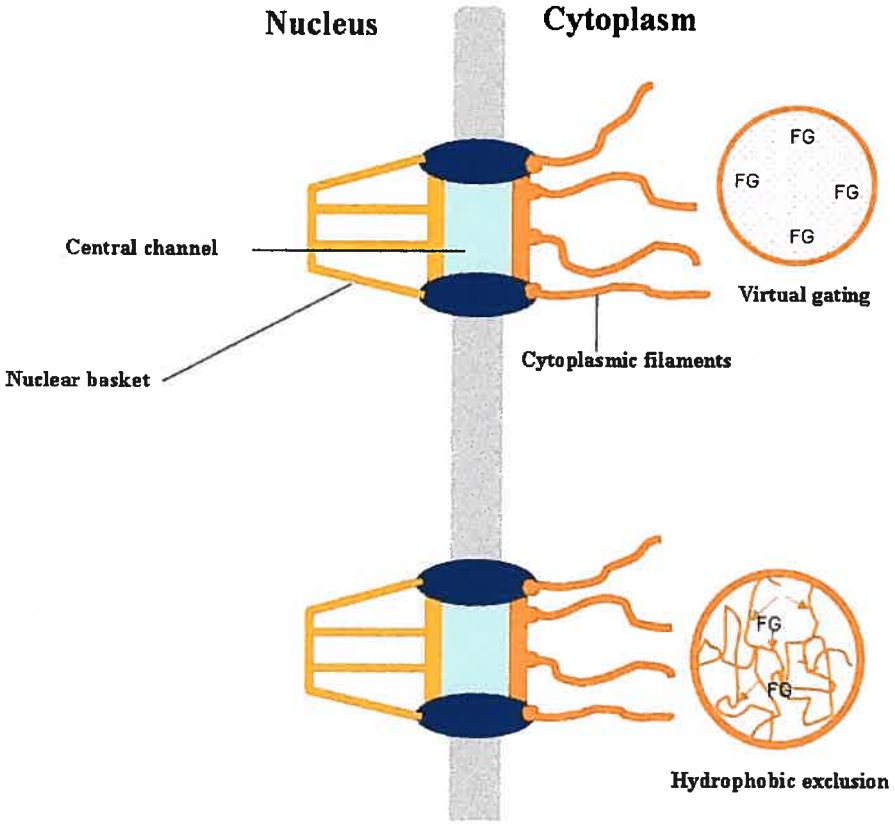


**Figure I.4. The nuclear pore complex (NPC).** The NPC is shown and the central channel through which translocation is thought to occur. The 'virtual gating' model suggests that the NPC consists of an aqueous channel and that the narrow constraints of this channel and the filamentous FG repeats of some nucleoporines function as an entropic barrier against diffusion. In contrast, the 'hydrophobic exclusion' model suggest the hydrophobic FG repeats weakly interact with each other to form a 'selective sieve'. Both models would facilitate the selective exclusion of proteins.

to deliver their genomes through the envelope of the interphase nucleus, which allows the infection of nondividing and terminally differentiated cells.

**Nuclear localization signals (NLSs)**: The most common mechanism employed by the cell to control molecules entering the nucleus is the use of nuclear localization signals (NLSs). The first signal sequence for nuclear import was identified in the simian virus 40 (SV40) large-T antigen, which consists of a short stretch of basic amino acids (PKKKRKV), designated as the basic type NLS.

Classic NLS are divided into two groups, monopartite including SV40 T antigen NLS and the more hydrophobic monopartite c-myc NLS (PAAKRVKLD). Another group is bipartite NLSs which consist of two stretches of basic amino acids separated by a spacer region, e.g. KRPAAIKKAGQAKKKK. Many viral capsid proteins contain NLSs which behave as nuclear import cargo, such as herpes simplex virus type 1 (HSV-1), parvovirus, influenza viruses and HIV-1 (O'Neill, Jaskunas et al. 1995; Wang, Palese et al. 1997; Haffar, Popov et al. 2000; Lombardo, Ramirez et al. 2002; Stelz, Rucker et al. 2002). However, although many capsid proteins may bind viral nucleic acid, there is very little information on the import of the actual protein-nucleic acid complex.

**Importin α:** Cytosolic proteins bearing a NLS are imported into the nucleus by the importin α/β heterodimer (or karyopherin α/β). Importin α (also known as Karyopherin α), a ~60kDa protein, is the import adaptor and can recognize and bind to the classical NLS on cytoplasmic proteins bound for the nucleus, then bind to the pore-docking protein (Adam, Adam et al. 1995). In *Saccharomyces serevisiae*, importin-α is called karyopherin-α, srp1p and KAP60, and is coded by the gene SRP1 (Pemberton, Blobel et al. 1998). The homologues in humans have been denoted hSRP1, hSRPα and hSRP1γ (Nachury, Ryder et al. 1998), importin-α1, α2 and α4 respectively (Herold, Truant et al. 1998). The basic importin α structure is a cylindrical super helix consisting of ten armadillo (ARM) repeats (Kobe, Gleichmann et al. 1999). Examination of the structure of importin-α reveals several interesting features: the NH2 terminal contains a basic stretch of highly conserved amino acid residues (10-55) comprising the importin-β binding (IBB) domain, the central NLS-binding domain with ten ARM repeats, and the C-terminus contains an acidic domain which binds to the cellular apoptosis susceptibility gene product (CAS), the function of which is to export importin α from the nucleoplasm (Fig.I.5) (Kutay, Bischoff et al. 1997; Herold,

Truant et al. 1998). The IBB domain serves a dual role. It binds to importin-β to

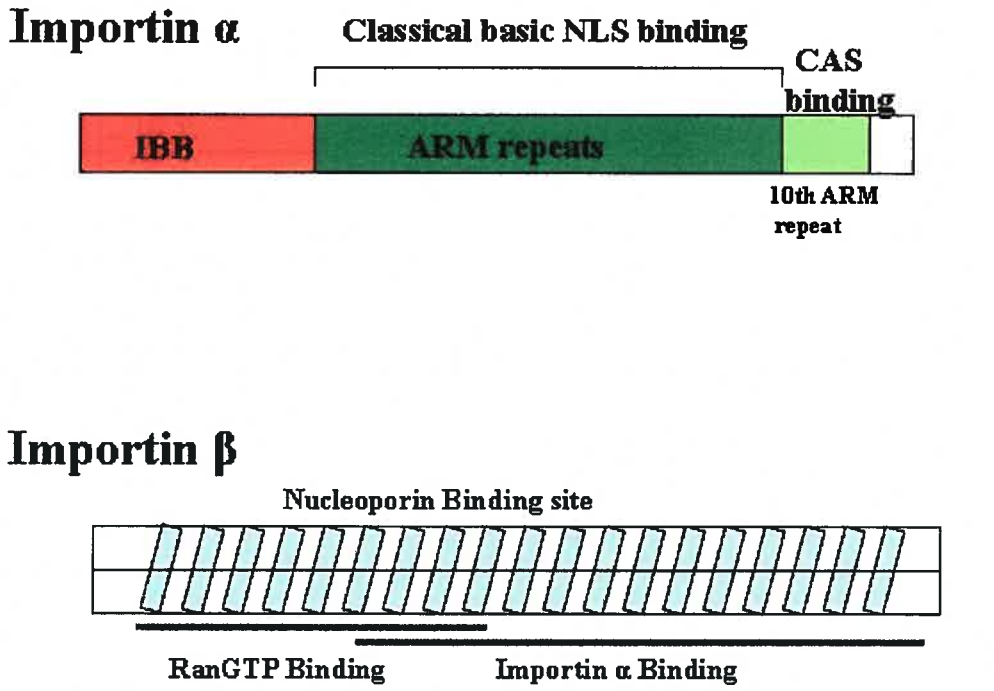


**Figure I.5. The domain structures of importin α and importin β.** Different functional domains and binding partners of importin α and importin β are indicated.

target the complex to the NPC for the translocation (Gorlich, Kraft et al. 1996; Moroianu, Blobel et al. 1996; Weis, Ryder et al. 1996) but it also can form an intramolecular interaction with the cNLS-binding pocket of importin α in the absence of cNLS cargo. A single round of importin α-mediated import can be divided into six steps, which include the formation of a ternary complex with cNLS cargo and importin β in the cytoplasm; importin β mediated binding of ternary complex to docking site at the periphery of the NPC and translocation through the NPC; dissociation of the ternary complex, triggered in part by the

binding of small nuclear GTPase RAN-GTP to importin β; recycling of importinα

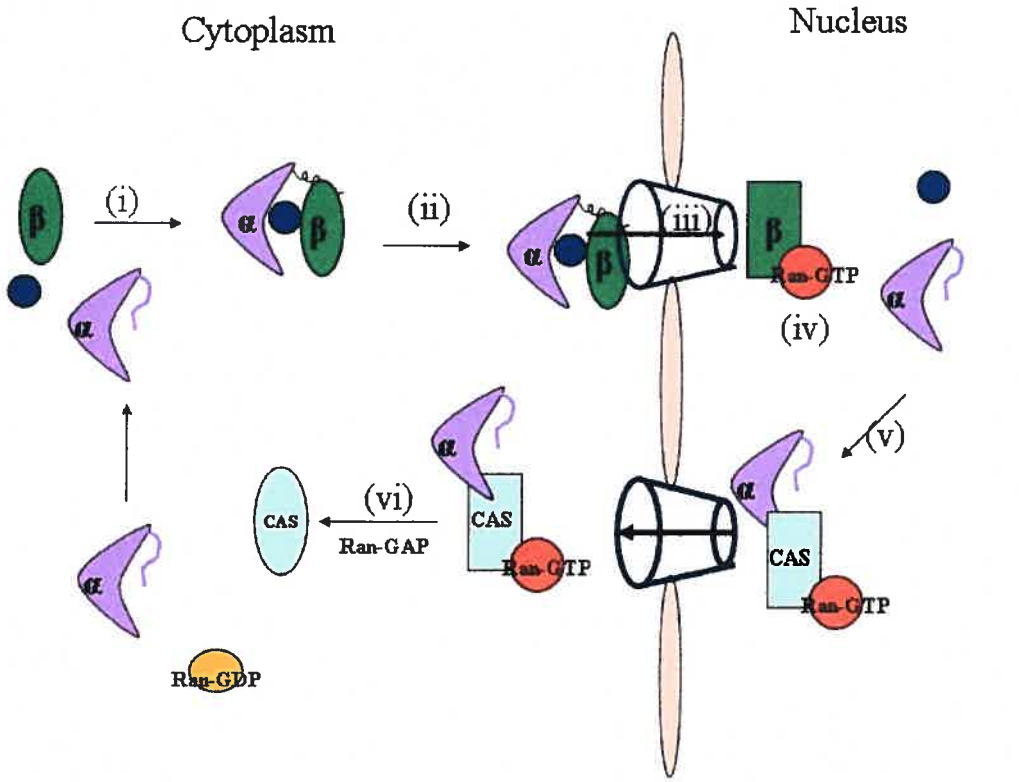


**Figure.I.6. The nucleocytoplasmic shuttling cycle of importin α.** (i) importin α forms a ternary complex with importin b and cargo (blue circles). (ii)The ternary complex docks at the NPC and (iii) translocates into the nucleus. (iv)Binding of Ran-GTP triggers the dissociation of the ternary complex. (v) importin α binds to the exportin CAS-Ran-GTP complex and is exported to the cytoplasm. (vi) Ran-GAP-stimulated hydrolysis of GTP by Ran triggers the dissociation of the exportin complex and releases free importin α into the cytoplasm from another transport cycle.

to the cytoplasm bound to the exportin CAS-RAN-GTP and finally release of free importin α to the cytoplasm (Fig.I.6.).

**Importin-β**: A 97 kDa import factor was found to bind to both importin-α and several xFxFG nucleoporins, revealing its role in docking the basic import complex to a docking site on the nuclear pore complex in an energy- and

temperature-independent process. Importin-β has been renamed importin-β1 or karyopherin-β1, since it has been found to be the first member of a large super family of importin-β homologues, with 14 members in yeast alone and more than 20 in mammalian cells (Pemberton, Blobel et al. 1998; Adam 1999). These receptors are generally large (90-130kDa) acidic proteins and contain multiple tandem helical repeats termed HEAT repeats (HEAT domains are named after the proteins in which similar sequences were first identified: huntingtin, elongation factor 3, the A subunit of protein phosphatase 2A, and the Tor1 kinase). All have in common an N-terminal Ran binding domain(Fig.I.5). Most of importin-β family members function in nuclear import (importins), nuclear export (exportins) or bidirectional nuclear transport. Recent studies have extended the role of importin-β to regulating the assembly of the nuclear envelope, mitosis and replication (Wiese, Wilde et al. 2001; Harel, Orjalo et al. 2003; Yamaguchi and Newport 2003).

Importin-β1 is unique among the importin-β family in its use of importin-α as an adaptor for binding substrates that contain the cNLS (Pemberton, Blobel et al. 1998). Other members of importin-β family bind their substrates directly. Importin-β1 can also import substrates such as retroviral proteins Rev and Tat in HIV, cyclin B1, and parathyroid homone-related protein (PTHrP) without binding importin-α (Lam, Briggs et al. 1999; Moore, Yang et al. 1999; Truant and Cullen 1999). Full-length importin-β1 consists of 19 HEAT repeats that form a compact super helical coil. Each HEAT repeat is made of two helices (A and B) connected by a loop. The A helices are located on the outside of the protein and form the convex face, whereas B helices, located inside, form the concave face (Cingolani, Petosa et al. 1999). The N-terminal (HEAT 1-10) and C-terminal (7-19) region bind RanGTP and IBB domain separately, whereas NPC binding sites occur on the convex face of importin-β(Fig.I.5). The overall protein coils into a short super

helix, with extensive interaction surfaces both on the inside and outer side of the super helix. Five proteins or protein fragments have been co-crystallized with β: a fragment of importin α (Cingolani, Petosa et al. 1999), RanGTP (Vetter, Arndt et al. 1999), a fragment of the transcription factor cargo protein SREBP-2 (Lee, Sekimoto et al. 2003), the PTHrP (Cingolani, Bednenko et al. 2002), and a short run of FG repeats from the yeast nucleoporin Nsp1(Bayliss, Littlewood et al. 2000). Although the structures for recognition of non-classic NLSs by importin β have not yet been determined, structural analyses show that each protein uses a different binding site on importin β and a different mode of interaction. In the structure of importin β1 complex with the NLS of PTHrP, the N-terminal set of HEAT repeats is used, enabling simultaneous binding of importin α and the NLS of PTHrP (Cingolani, Bednenko et al. 2002). Another structure of importin β1 bound to SREBP-2, a helix-loop-helix zipper (HLHZ) transcription factor, shows that HLHZ domain requires importin β1 to adopt a more open confirmation and to use more hydrophobic interactions than in its complexes with importin α or PTHrP (Lee, Sekimoto et al. 2003). It appears that the spring-like super helical importin β has an inherently large degree of flexibility (Stewart 2003). Therefore, it is clear that, by using different binding sites combined with adopting distinct conformations, one importin β can recognize more than one type of NLSs which it uses to recognize its many partners.

**Ran and the termination of transport**: After translocation though the NPC, importin -cargo complexes encounters RanGTP. This interaction is crucial for termination of the transport cycle. In the case of import receptor, RanGTP binding in the nucleus triggers dissociation of the import receptor-cargo complex, thus releasing cargo into the nucleus, and the import receptors return to the cytoplasm as RanGTP-receptor complexes (Fig.I.6). The Ran GTPase cycle is a key to promoting the directionality of nuclear transport. Ran is an extremely

abundant and soluble GTPase localized predominantly inside the nucleus

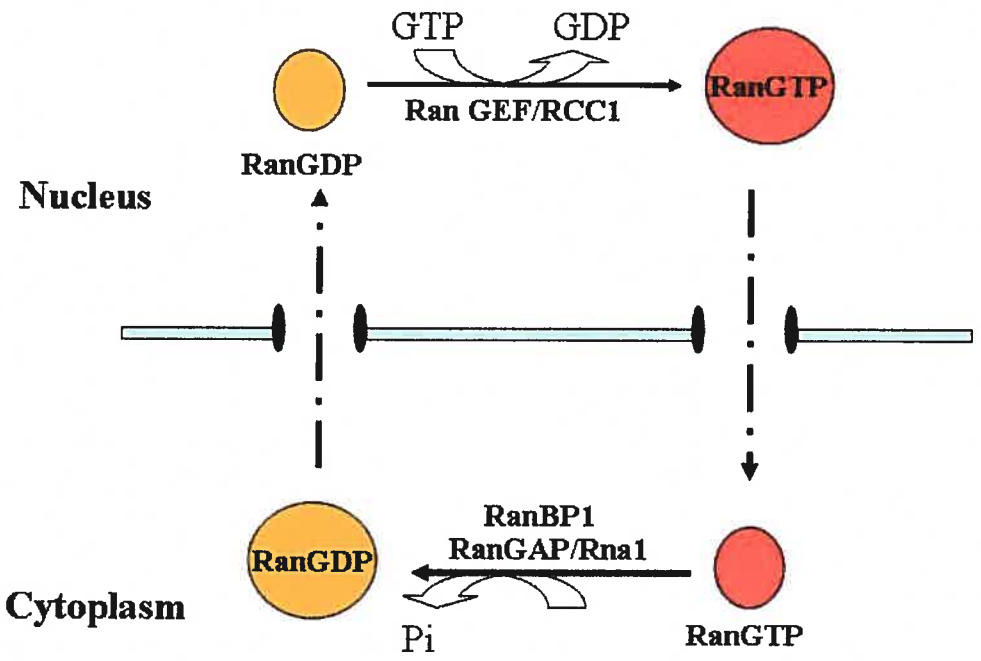


**Figure I.7. The Ran GTPase Cycle.** Ran is maintained as RanGTP in the nucleus by the activity of the Ran guanine nucleotide exchange factor (RanGEF or Rcc1) and as RanGDP in the cytoplasm by the Ran GTPase activating protein (RanGAP or Rna1p) associated with Ran-binding protein1 (RanBP1).

at steady state, and, like all small GTPases, it has very low nucleotide hydrolysis and exchange activity on its own (Melchior and Gerace 1998; Moore 1998). Ran's associations with different cellular proteins are dependent upon the nucleotide that is bound to it. Regulatory proteins increase the rates of nucleotide exchange and nucleotide hydrolysis, allowing Ran to cycle between the GTP- and GDP-bound states at a physiological rate. A guanine nucleotide exchange factor (RanGEF) promotes release of guanine nucleotides, allowing Ran to acquire GTP. RanGEF is localized within nuclei throughout interphase and is bound to chromatin. A Ran GTPase activating protein (RanGAP) stimulates nucleotide

hydrolysis. RanGAP and its coactivator, Ran-binding protein 1 (RanBP1), are localized in the cytosol during interphase (Matunis, Coutavas et al. 1996; Mahajan, Delphin et al. 1997) (Fig.I.7). The localization of Ran's regulators would suggest that Ran is primarily GTP-bound in the nucleus and GDP-bound in the cytoplasm. It would also suggest that Ran moves between these compartments in order to undergo a complete round of GTP binding and hydrolysis.

**Crossing the channel:** How does the karyopherin-cargo move through the NPC? One of the most important functional requirements for importin β in mediating nuclear transport is their ability to bind specially to many nucleoporin that contain consecutive FG-repeats (Radu, Moore et al. 1995). Several models have been proposed to explain the mechanism of NPC gating and karyopherin-cargo translocation through the NPC (Weis 2003).

In the "virtual gating" model, the NPC consists of an aqueous channel, and the narrow constraints of this channel and the filamentous FG repeats function as entropic barriers to diffusion. Binding of Kaps to the FG repeats enables them to overcome the entropic barrier, to access the channel and to diffuse through the NPC (Fig.I.4) (Rout, Aitchison et al. 2000; Rout, Aitchison et al. 2003). The second model suggests that the hydrophobic FG repeats interact weakly with each other to form a 'selective sieve'. These interactions enable the selective exclusion of proteins that cannot dissolve into the sieve, but the hydrophobic nature of Kaps enables them to enter the sieve and cross the NPC (Fig.I.4) (Ribbeck and Gorlich 2002). Another model suggests that Kaps travel along an affinity gradient of Nup-binding sites, encountering Nups of increasing affinity during translocation (Ben-Efraim and Gerace 2001; Pyhtila and Rexach 2003).

Overall, the common properties that define a karyopherin directly delineate the transport mechanism: substrate binding, interaction with Nups, cytoplasmic-nuclear shuttling and RanGTP binding.

### 1.2.3 Nuclear Import of Viral DNA Genomes

**Nuclear entry of DNA viruses**: Viruses have found multiple strategies to deliver this incoming genome into the nucleus. Large DNA viruses, such as adenoviruses (Ad) and herpes simplex virus (HSV), dissociate the genome from the capsid prior to nuclear import. The Ad particles directly dock to the NPC protein CAN/Nup214, located at the cytoplasmic filaments (Wisnivesky, Leopold et al. 1999; Trotman, Mosberger et al. 2001). At this position, a series of disassembly factors are recruited, including the nuclear histone H1, the H1 import factors importin β and importin 7, and the heat shock protein Hsp70. These factors facilitate the spatially controlled capsid disassembly at the NPC and are required for import of the viral genome (Saphire, Guan et al. 2000). In contrast to Ad, after importin β-mediated NPC docking, HSV-1 capsid injects DNA into the nucleus by cytosol-dependent way (Dasgupta and Wilson 1999; Ojala, Sodeik et al. 2000). Small DNA viruses are thought to maintain their genome in an encapsulated state until they arrive in the nucleus. Parvovirus and hepatitis B virus (HBV) capsids are smaller than 35nm in diameter. In the case of autonomous parvovirus minute virus of mouse (MVM) and human HBV, the cytosolic capsids undergo conformational changes that expose NLSs and enable capsid interactions with importins. This increases the affinity to the NPC, and leads to viral capsid translocation through the NPC. The mechanisms of capsid disassembly in the nucleoplasm are unknown (Lombardo, Ramirez et al. 2000; Mabit, Breiner et al. 2001; Lombardo, Ramirez et al. 2002; Vihinen-Ranta, Wang et al. 2002).

**Nuclear entry of RNA viruses**: Influenza viruses are enveloped animal viruses with a segmented, negative-sense RNA genome and need to enter the nucleus to make use of the nuclear splicing machinery. The nucleoprotein (NP) constitutes the major protein component of the viral ribonucleoprotein (vRNP) and it forms a

proteinaceous core around which the RNA is wrapped in a helical fashion. Available evidence shows that helical vRNPs enter the nucleus in an intact form through nuclear pores (Martin and Helenius 1991; Kemler, Whittaker et al. 1994). The length of the individual vRPNs varies from 20 to 80 nm depending on the size of the RNA. However, the diameter of the rod-shaped particles is small enough (10-20nm) to allow active passage of vRNPs if they move lengthwise through the pore. NP is generally though to enter the nucleus using basic-type NLSs that localize at its N-terminus and interaction with importin α (O'Neill, Jaskunas et al. 1995; Wang, Palese et al. 1997; Stevens and Barclay 1998).

For access to the nucleus, most retroviruses rely on disassembly of the nuclear envelope during mitosis. Onco-retroviruses, like the Moloney murine leukemia virus (MoMLV), can infect only dividing cells, while avian retroviruses, such as Rous sarcoma virus, are partially restricted to dividing cells. Human foamy virus might have some ability to infect cells in interphase, although there is controversy on this issue (Roe, Reynolds et al. 1993; Lewis and Emerman 1994; Saib, Puvion-Dutilleul et al. 1997; Hatziioannou and Goff 2001). In contrast, the lentiviruse, such as HIV-1, infect dividing and nondividing cells by using an active nuclear import pathway (Bieniasz, Weiss et al. 1995). This topic will be discussed in more detail in Part I.3.

## I.3 HIV-1 PIC NUCLEAR IMPORT.

HIV-1 life cycle requires the integration of viral DNA into the host cell's genome to form provirus. To achieve this, the viral reverse-transcribed DNAs must enter the nucleus as a part of a large preintegration complex (PIC). The size of retroviral PICs is estimated to be roughly equivalent to the size of eukaryotic ribosome such that they cannot be transported passively into the nucleus through nuclear pore complexes (NPCs) (Vodicka 2001; Greene and Peterlin 2002). However, the lentiviruses such as HIV-1 have evolved sophisticated mechanism the ensure efficient transport of their genome into the nucleus of non-dividing

cells, including macrophages and quiescent T lymphocytes (Gartner and Popovic 1990; Weinberg 1991). It is known that HIV-1 is able to infect non-dividing cells because its PIC has a mitosis-independent nuclear import capability (Bukrinsky, I.N. et al. 1992; Lewis 1992; Lewis and Emerman 1994; Bukrinsky and Haffar 1998; de Noronha, Sherman et al. 2001; Le Rouzic, Mousnier et al. 2002). This ability is one of the hallmarks that distinguishes HIV-1 from oncoretroviruses (Weinberg 1991; Lewis 1992; Roe, Reynolds et al. 1993; Lewis and Emerman 1994). Particularly, this feature has been shown to be important for the establishment of HIV-1 replication and pathogenesis in exposed hosts, since the infection of post mitotic cells including tissue macrophages, mucosal dendritic cells and quiescent T cells is essential not only for viral transmission and dissemination, but also for the establishment of persistent viral reservoirs (Meltzer, Skillman. D.R. et al. 1990; Innocenti, Ottmann et al. 1992; Ho, Cherukuri et al. 1994).

At the molecular level, the ability of HIV-1 to infect non-dividing cells has been attributed to the karyophilic properties of HIV-1 PICs. Although a detailed compositional and stoichiometric description of HIV-1 PICs has yet to be attained, a number of viral proteins, including IN, matrix (MAp17gag), Vpr, and RT, have been identified to be associated with this nucleoprotein complex. There are several possibilities for the molecular mechanisms of PIC nuclear import. First, proteins that remain associated with the viral genome after uncoating may mediate PIC import into the nucleus, and this may be assisted by specific cellular factors. Interestingly, three viral proteins, MA, IN and Vpr, have been proposed to play significant roles in HIV-1 nuclear import (Bukrinsky, Haggerty et al. 1993; Heinzinger, Bukinsky et al. 1994; Gallay, Swingler et al. 1995; Gallay, Swingler et al. 1995; Yao, Subbramanian et al. 1995; Gallay, Hope et al. 1997; Vodicka, Koepp et al. 1998; Zhou, Lu et al. 1998; Zennou, Petit et al. 2000). Second, the retroviral genome itself may contain nuclear targeting elements. Recently, a unique DNA structure, named the central DNA flap, was also shown to play a role

in PIC nuclear import (Zennou, Petit et al. 2000). The third possibility has been raised by the observation that HIV-1 Vpr causes transient bulges in the nuclear envelope and thus might locally open the gate for nuclear entry of PIC independent of NPC (de Noronha, Sherman et al. 2001).

**Factors that effect PIC entry into nucleus**

**I.3.1 Matrix (MA):** MA was the first protein implicated in HIV-1 nuclear import in non-dividing cells (Bukrinsky, Haggerty et al. 1993). MA was originally shown to contain a sequence at its N-terminus similar to basic NLS ($^{25}$GKKKYKLKH) (Bukrinsky, Haggerty et al. 1993). It was subsequently shown that recombinant GST-MA fusion protein, as well as MA in the uncoated viral nucleoprotein complex, can bind importin $\alpha$ and that binding only occurs when there is an intact N-terminal basic domain (Gallay, Stitt et al. 1996). However, several later reports questioned the role of MA in HIV-1 nuclear import as viruses lacking MA-NLS still replicate in non-dividing cells even at reduced levels (Freed, Englund et al. 1995; Fouchier, Meyer et al. 1997; Reil, Bukovsky et al. 1998). Interestingly, a following study identified a second NLS in the C-terminal region of MA, and functional analysis revealed that mutations of both MA NLSs resulted in impairment of nuclear import in non-dividing cells (Haffar, Popov et al. 2000). A nuclear export signal (NES) was also identified in MA, since the fusion protein MA-GFP is redistributed from the cytoplasm to the nucleus in the presence of leptomycin B (an inhibitor of nuclear export) (Dupont, Sharova et al. 1999; Depienne, Roques et al. 2000). It was suggested that the C-terminal tyrosine phosphorylation of MA also plays a role in the regulation of the localization and nuclear import of the PIC (Gallay, Swingler et al. 1995). Thus, while MA is required for efficient nuclear import of HIV-1 PIC, the mechanism is not clear and its role appears to be nonessential. It is very likely to be only one of several factors regulating this process.

**I.3.2 Vpr**: Vpr is another HIV-1 encoded protein associated with viral PIC nuclear import in non-dividing cells (Heinzinger, Bukinsky et al. 1994; Connor, Chen et al. 1995; Nie, Bergeron et al. 1998; Vodicka, Koepp et al. 1998). Vpr is a 96-amino acid polypeptide that is packaged into progeny virions through its interaction with the C-terminal $p6^{Gag}$ domain of $Pr55^{Gag}$ precursor protein. It is localized predominantly in the nucleus and at the nuclear envelope (Vodicka, Koepp et al. 1998; Kamata and Aida 2000). Deletion of Vpr decreased transport of the viral genome to the nucleus as determined by 2-LTR circle and decreased infection of macrophages (Heinzinger, Bukinsky et al. 1994). However, while no conventional NLS is detectable in Vpr, two independent signals, one in the N-terminal α helical region and the other in the carboxyl half of the protein, have been implicated in Vpr nuclear import (Lu 1993; Di Marzio, Choe et al. 1995; Yao, Subbramanian et al. 1995; Jenkins, McEntee et al. 1998; Zhou, Lu et al. 1998; Sherman, de Noronha et al. 2001). Jenkins *et al.* reported that the Vpr nuclear localization pathway appears to be distinct from the classical NLS- and M9-dependent nuclear import pathways (Jenkins, McEntee et al. 1998), while others found that Vpr directly interacts with importin α (Popov, Rexach et al. 1998; Vodicka, Koepp et al. 1998; Kamata, Nitahara-Kasahara et al. 2005) and nucleoporin hCG1 (Fouchier, Meyer et al. 1998; Le Rouzic, Mousnier et al. 2002). Interestingly, it was shown that binding of Vpr to importin α increased the affinity of MA interacting with importin α, suggesting that the formation of such a trimetric complex may increase the karyophilic potential of the HIV-1 PICs (Popov, Rexach et al. 1998). More recently, Vpr expression has also been shown to induce transient bulges in the nuclear envelope, which may create a channel between the nucleus and the cytoplasm and may facilitate HIV-1 PIC nuclear import (de Noronha, Sherman et al. 2001). Such a model proposes that, after reverse transcription, the PICs remain cytosolic, but in close proximity to the nuclear envelope. Vpr would dissociate from the PIC and enter the nucleus. The

nuclear Vpr could then induce bursting and resealing of the nuclear envelope, with the concomitant entrapment of PICs within the nucleus. However, since HIV-1 is not a typical lytic virus, it is uncertain if the Vpr-mediated nuclear envelope disruption is enough to allow PIC passage. Moreover, while Vpr plays an important role in HIV replication in tissue macrophages, it does not appear to have a significant role in HIV growth in cycling T cells, PBMC, naïve T cells and growth-arrested indicator cells (Bouyac-Bertoia, Dvorin et al. 2001; Eckstein, Sherman et al. 2001).

**I.3.3 Integrase**: Viruses lacking Vpr and MA-NLS still replicate in non-dividing cells, although at reduced levels, and so another viral determinant was sought. Gallay *et al.* first showed that HIV-1 IN was able to localize in the nucleus and associate with importin α in an *in vitro* binding assay, suggesting that IN may contribute to viral nuclear import (Gallay, Hope et al. 1997). Consistently, the karyophilic feature of IN was further confirmed by different groups using various IN-fusion proteins, including GFP-IN, BSA-IN and pyruvate kinase (PK)-IN fusions (Pluymers, Cherepanov et al. 1999; Petit, Schwartz et al. 2000; Tsurutani, Kubo et al. 2000; Bouyac-Bertoia, Dvorin et al. 2001; Depienne, Mousnier et al. 2001; Limon, Devroe et al. 2002; Lu, Limon et al. 2004). Moreover, the contribution of IN to HIV-1 nuclear import has been documented in several reports (Bouyac-Bertoia, Dvorin et al. 2001; Ikeda, Nishitsuji et al. 2004). Interestingly, even though the nature of the pathway used by IN was not known, these studies found that the nuclear import function of IN is essential for productive HIV-1 infection of both dividing and non-dividing cells. This unexpected result suggests that the nuclear entry of HIV-1 PICs in dividing cells may not be a passive process. Consistent with this, it was reported that the nuclear import of HIV-1 PICs might be mitosis-independent in cycling cells (Katz, Greger et al. 2003).

In an attempt to characterize the karyophilic feature of IN, a study by Gallay *et al.* suggested that two regions in IN ($^{186}$KRK and $^{211}$KELQKQITK) may constitute a bipartite NLS motifs since mutants K186Q and Q214/216L in these regions lost the protein nuclear localization and their ability to bind to karyopherin α *in vitro* (Gallay, Hope et al. 1997). However, while the study by Petit *et al.* confirmed their nuclear localization results (Petit, Schwartz et al. 2000), other researchers could not prove the importance of these mutants for protein nuclear localization and/or their roles in viral nuclear import; rather they appear to contribute to reverse transcription and/or integration (Petit, Schwartz et al. 2000; Tsurutani, Kubo et al. 2000; Bouyac-Bertoia, Dvorin et al. 2001; Lu, Limon et al. 2004). Moreover, while Bouyac-Bertoia *et al.* reported a noncanonical NLS within the catalytic core domain of IN (Bouyac-Bertoia, Dvorin et al. 2001), later reports were unable to confirm this observation (Dvorin, Bell et al. 2002; Limon, Devroe et al. 2002). In addition, the mechanism(s) involved in IN nuclear localization is also controversial. By using *in vitro* binding assays, several studies have shown that IN interacts with importin α (Gallay, Hope et al. 1997; Fassati, Gorlich et al. 2003; Armon-Omer, Graessmann et al. 2004), while Depienne *et al.* revealed that IN nuclear accumulation *in vitro* neither involve importin α, β1, and β2-mediated pathways, nor GTP hydrolysis (Depienne, Mousnier et al. 2001). Moreover, other studies have implicated nuclear translocation of IN to nucleus by its interaction with a cellular component LEDGF/p75 (Cherepanov, Maertens et al. 2003; Maertens, Cherepanov et al. 2003). However, recent studies revealed that LEDGF/p75-IN interaction appears to be essential to tether IN to host chromosomes for viral DNA integration and to protect it from proteasomal degradation, rather than to IN nuclear translocation (Llano, S. et al. 2004; Llano, Vanegas et al. 2004; Emiliani, Mousnier et al. 2005).

Based on similarity to importin β, a family of nuclear import receptors, including importin 7 (Imp7), were discovered and shown to contribute

specifically to nuclear transport of a wide variety of proteins and RNAs into and out of the nucleus (Pollard, Michael et al. 1996; Gorlich, Dabrowski et al. 1997; Gorlich and Kutay 1999). Imp7 (formerly called RanBP7) was initially identified as one of importin receptors which can mediate nuclear import of ribosomal proteins in mammalian cells (Gorlich, Dabrowski et al. 1997; Jakel and Gorlich 1998). Unlike other importins, such as importin 5, that is in sequence clearly related to Impβ, Imp7 is more distantly related, and significant homology to impβ is restricted to the N-terminal Ran-binding domain (Gorlich, Dabrowski et al. 1997). More recent studies have also demonstrated that Imp7 is involved in nuclear translocation of the glucocorticoid receptor (Freedman and Yamamoto 2004) and Imp7 can form a heterodimer complex with Impβ, that is a functional complex for bind to histone H1 and mediate its nuclear import (Jakel, Albig et al. 1999; Bauerle, Doenecke et al. 2002). Intriguingly, a recent study by Fassati *et al.* showed that Imp7 mediates HIV-1 PIC nuclear import *in vitro* (Fassati, Gorlich et al. 2003). Their study also suggested that the action of Imp7 in HIV-1 PIC nuclear import may be through its binding to HIV-1 IN, since their *in vitro* binding assay indicate that recombinant IN could pull down Imp7 from HeLa cell lysates (Supplementary materials in (Fassati, Gorlich et al. 2003). Moreover, in the same system, they also showed that IN was able to pull down Impα, Impβ and transportin. Therefore, it is still unclear whether the co-pull down of different karyopherins with IN was through their direct interaction or derived from a nuclear import complex. Overall, even though extensive studies have been dedicated to this specific research field, the contribution of HIV-1 IN to viral PIC nuclear import remains to be defined.

**I.3.4 The central DNA flap**: HIV-1 nuclear import has also been found to require structural determinants present in the viral DNA. The reverse-transcribed HIV-1 genome contains a short triple-stranded overlap (the central DNA flap), which is formed during plus-strand DNA synthesis. Plus strand DNA is synthesized as two

discrete half-genomic segments. A central copy of the polypurine tract cis-active sequence (cPPT, over overlaps with the integrase coding gene), present in all lentiviral genomes, initiates synthesis of a downstream plus strand. The upstream plus strand segment initiated at the 3'PPT will, after a strand transfer, proceed until the center of the genome and terminate at the central termination sequence (CTS), which ejects HIV-1 RT at this site. Thus, the final product of HIV-1 reverse transcription is a linear DNA bearing in its center a 99-nucleotide plus strand overlap, the central DNA flap (Fig.I.8). Early studies suggested that Mutations in the cPPT or CTS severely impair HIV replication (Charneau, Alizon et al. 1992; Charneau, Mirambeau et al. 1994). Subsequent studies have revealed that the central DNA flap acts as a cis-determinant of HIV-1 DNA nuclear import (Zennou, Petit et al. 2000). Zennou and colleagues engineered an HIV genome that contained mutations in the cPPT while otherwise maintaining reverse transcription and the integrity of the integrase gene. This DNA flap mutant of HIV was impaired in single-round infection assays and replication was reduced in both dividing and growth-arrested cells. These mutant HIV-1 genomes accumulated at the vicinity of the nuclear membrane. Finally, the investigators showed that by inserting a central cis-acting DNA flap into an HIV-based vector lacking a PPT, one could greatly enhance the infection of growth-arrested cells. Other studies also have reported that the central DNA flap conferred an infection advantage of approximately 2 to 10 fold on single-round HIV-1-derived lentiviral vectors, thus suggesting that the central DNA flap facilitated early step(s) of lentiviral transduction (Parolin, Taddeo et al. 1996; Follenzi, Ailles et al. 2000; Sirven, Pflumio et al. 2000; Dardalhon, Herpers et al. 2001; Park and Kay 2001; Zennou, Serguera et al. 2001; Maele, Rijck et al. 2003). However, following studies provided evidence that the central DNA flap did not play a role in either PIC nuclear import or HIV-1 replication, as the reduction of replication is rather small and seems to be strain dependent (Dvorin, Bell et al. 2002) (Limon,

Nakajima et al. 2002). Thus, the exact impact of the central DNA flap on the HIV-1 infection is still an open question that remains to be clarified.

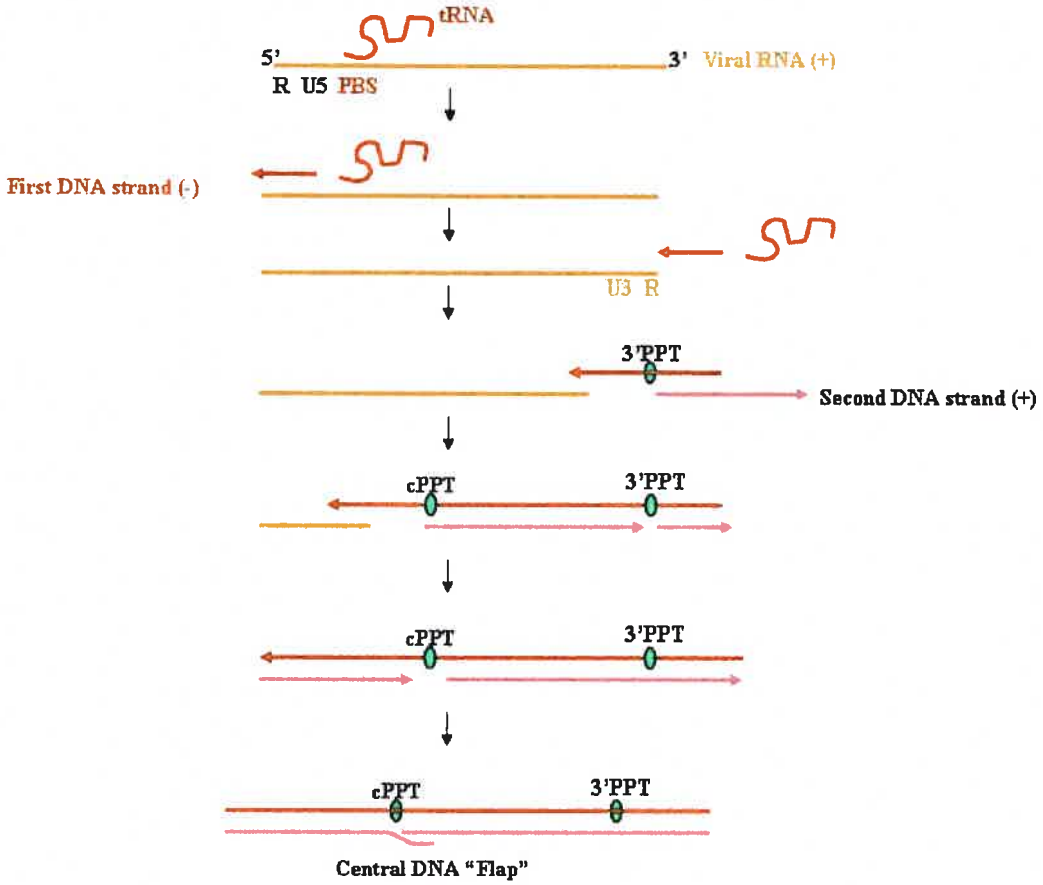


**Figure I.8. A schematic representation of the reverse-transcription process of HIV-1 RNA.** The generation of the central DNA FLAP in HIV-1 cDNA is repersentated. PBS, primer-binding site; cPPT, central polypurine tract; 3'PPT, 3' polypurine tract.

Altogether, even though much work trying to explain the importance of several viral elements such as MA, Vpr, IN and central DNA flap for the ability of lentiviruses to infect non-dividing cells, the results are controversial and none of the described NLSs in those elements seems to be essential for infection of non-dividing (Fouchier, Meyer et al. 1997; Freed, Englund et al. 1997; Dvorin, Bell et al. 2002; Limon, Nakajima et al. 2002; Petit, Schwwartz et al. 2000; Reil,

Bukovsky et al.1998; Yamashita and Emerman, 2005). It is possible that the effect of the different NLS are redundant, and therefore HIV still retained some ability to infect non-dividing cells because of the presence of other NLS on other proteins. Another explanation is that events in the viral lifecycle other than nuclear import may be more important in determining the ability of a given retrovirus to infect non-dividing cells. The association of CA with incoming virions is different for MLV than it is for HIV since capsid (CA) is tightly associated with intracellular complexes of MLV, but not HIV. Yamashita et al. suggested that CA is a dominant determinanat of retrovirus infectivity in nondividing cells (Yamashita and Emerman, 2004). They hypothesized that uncoating, rather than nuclear import might be the rate-limiting step in the ability to infect non-dividing cells if incoming virions can enter the nucleus only after uncoating has proceeded (Yamashita and Emerman, 2006; Dismuke et al. 2006). So, the possible model is that CA alters the accessibility of the PIC to cellular or viral factors that determine nuclear entry and thereby determines the fate of nuclear transport in nondividing cells.

### I.4 Integrase and Integration

Integration of retroviral DNA into the host genome is a distinguishing feature and essential step of HIV-1 replication and is mediated by viral IN, a 32-KD protein generated by protease-mediated cleavage of the C-terminal portion of the HIV-1 Gag-Pol polyprotein. Integration can be subdivided into two steps: (i) "3'-end processing". In the cytoplasm, IN removes two or three nucleotides from the initially blunt 3' termini of both strands of full-length, linear viral DNA, generating a preintegration substrate with 3'-recessed ends; (ii) "strand transfer". In the nucleus, IN couples the 3'-recessed DNA ends to the 5'overhanging termini of the cleaved cellular DNA. Cellular repair machinery completes the integration process by removing the unpaired dinucleotides from 5'-ends of the viral DNA

and fills the gap between viral and target DNA (Chow, Vincent et al. 1992). For the integration reaction, divalent metal ions such as $Mn^{2+}$ or $Mg^{2+}$ are required but no source of energy is needed (d'Angelo, Mouscadet et al. 2001). Purified IN can carry out "3'-end processing" and strand transfer reaction *in vitro* when combined with short synthetic oligonucleotides that mimic the viral DNA ends and a divalent metal ion. IN also catalyzes a reaction, known as disintegration, which is essentially the strand transfer reaction in reverse (Chow, Vincent et al. 1992).

### I.4.1 The structure of HIV-1 IN

HIV-1 IN proteins are composed of three structurally and functionally distinct domains: an N-terminal, zinc-finger-containing domain (residues 1-50, NTD), a core domain (residues 50-212, CCD), and a relatively nonconserved C-terminal domain (residues 212-288, CTD) (Fig.I.9.). Unfortunately, the complete three-dimensional bioactive structure of HIV-1 integrase is still unknown because of its low solubility. However, the structures of the three separate domains have been solved by X-ray crystallography or NMR spectroscopy. Structures also exist for the core domain plus N-terminal domain (Wang, Ling et al. 2001) and for core domain plus the C-terminal domain (Chen, Krucinski et al. 2000).

**The core domain of IN (CCD):** There are three highly conserved residues in the central core domain of HIV-1 IN, $Asp^{64}$, $Asp^{116}$, and $Glu^{152}$ (the D,D-35-E motif) (Fig.I.9). Mutation any one of these residues block the enzyme activity, demonstrating their key role in catalysis. The core domain alone can catalyze the disintegration reaction, but both N- and C-terminal domains are required for 3'-end processing and strand transfer (Schauer and Billich 1992; Bushman, Engelman et al. 1993; Engelman, Bushman et al. 1993; Vink, Oude Groeneger et al. 1993; Mazumder, Engelman et al. 1994; Kulkosky, Katz et al. 1995). A systematic replacement of the hydrophobic residues resulted in a mutant, F185K, which had considerably improved solubility of the core domain and led to its

crystal structure (Jenkins, Hickman et al. 1995). The central feature of the structure is a five-stranded β sheet flanked by six helical regions (Fig.I.10). Two core domains associate to form a two-fold axis related dimer. This domain of IN belongs to a superfamily of polynucleotide transferases that includes RNaseH and the bacteriophage MuA transferase (Rice and Mizuuchi 1995). The loop (residues 140-152) that is adjacent to the active site region is flexible and is quite difference in many of the structures (Dyda, Hickman et al. 1994).

**The N-terminal domain of IN (NTD)**: The N-terminal domain (1-50) of HIV-1 IN contains a highly conserved HHCC (H12, H16, C40, C43) motif, which binds one equivalent of zinc, stabilizing the interaction between the helices and overall IN structure, as well as promoting the formation of higher-order IN multimers (Zheng, Jenkins et al. 1996). Mutation of the two cystines in the HHCC motif in HIV-1 IN also affects 3'-processing and strand transfer (Engelman and Craigie 1992). The Structure of the N-terminal domain of HIV-1 IN is highly α-helical, with the monomer consisting of four helices. A hydrophobic core stabilizes the upper region of the structure while the lower region is stabilized by $Zn^{2+}$ coordination. The structure of the N-terminal plus core domains (residues 1-212) for an HIV-1 triple mutant (W131D, F139D, F185K) has been determined (Wang, Ling et al. 2001). The crystals contain four monomers per symmetric unit. The linker region joining the N-terminal and core domains (residues 47-55) is disordered in all four structures.

**The C-terminal domain of IN (CTD)**: The C-terminal domain has five strands arranged antiparallel to form a β barrel which adopts an SH3-like fold and has been shown to bind DNA non-specifically (Vink, Oude Groeneger et al. 1993; Lodi, Ernst et al. 1995; Eijkelenboom, Sprangers et al. 1999). The crystal structure of the two domain HIV-1 integrase, residues 52-288 has also been determined by X-ray crystallography (Chen, Krucinski et al. 2000). Two C-

terminal domains are related to each other by 90° rotation relative to their two-

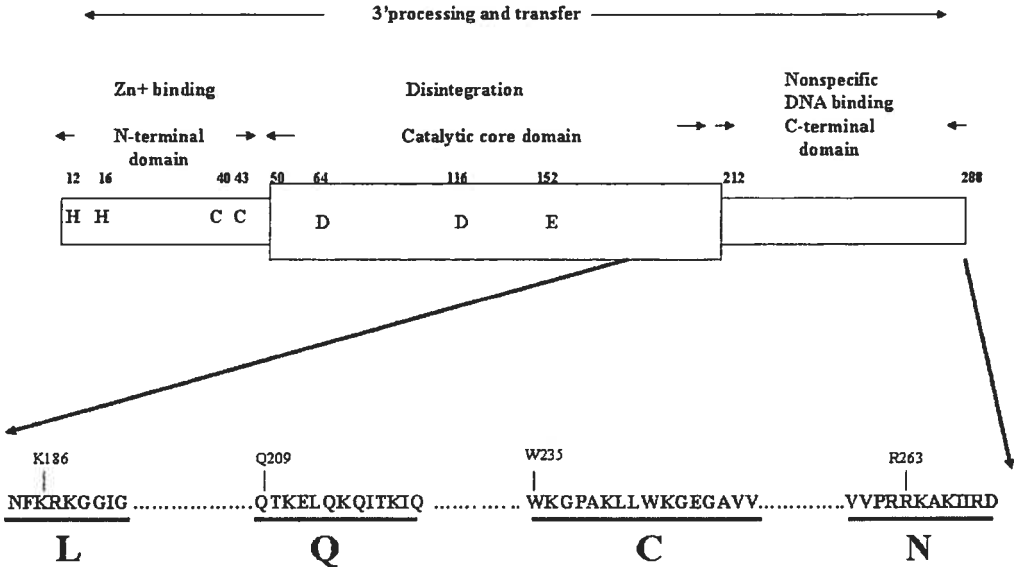


**Figure.I.9. Three domains of IN and Conserved sequences in the carboxyl terminus of retroviral IN.** Region L is only conserved in the lentiviruses; region C and N are conserved in all retroviruses, although the consensus sequences differ between the lentiviruses and the nonlentiviruses. Q is glutamine-rice/basic region in the lentiviruses. The polypurine tract cis-active sequence (cPPT) and the central termination sequence (CTS) are located at the L and Q region.

fold axis. Within the dimer, the catalytic core domains form the only dimer interface, and the C-terminal domains are located 55 A° apart. A 26-aa α-helix, α6, links the C-terminal domain to the catalytic core.

### I.4.2 Characterization of the DNA-binding domain of HIV-1 IN.

Current experimental evidence from photocrosslinking and mutagenesis suggest that all three domains interact with DNA: The core domain is responsible for sequence-specific recognition of the last six base pairs of viral LTR ends (LaFemina, Callahan et al. 1991; Ellison and Brown 1994; Jenkins, Esposito et al1997; Esposito and Craigie 1998; Gao, Butler et al. 2001). The C- domain is

responsible for nonspecific binding of more distal bases of the LTRs

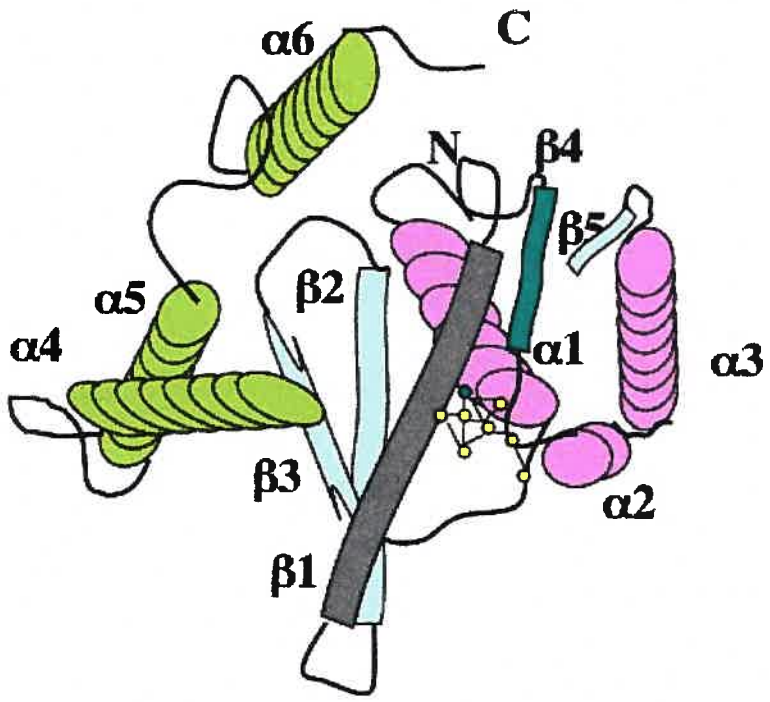


**Figure I.10.** Ribbon diagram of the core domain of HIV-1 IN. β strands and α helices are marked. Two residues of the D,D-35-E motif, $Asp^{64}$ and $Asp^{116}$, are shown as ball-and-stick models.

(Jenkins, Esposito et al. 1997; Esposito and Craigie 1998; Heuer and Brown 1998). The N-terminal domain is in close proximity to target DNA 5' to the site of integration (Heuer and Brown 1998) but the first 26 residues of HIV-1 IN are not required for DNA binding (Mumm and Grandgenett 1991; Mazumder, Neamati et al. 1996). More recently, Luca L.D. *et al* generated the three-domain protein-viral DNA complex through an automated docking algorithm (De Luca, Vistoli et al. 2005). They suggested that the viral DNA interacted with all three IN domains and the amino acids which formed direct interactions with the viral DNA were residues K156, K159, K160, K186, K188 in the catalytic core domain of chain B,

R20 in the N-terminal domain of Chain B, and S230, R231, W244, K263, and K264 in the C-terminal domain of Chain A.

Numerous studies have analyzed HIV-1 mutants altered at IN active-site residues (the D,D-35-E motif) that result in class I replication-defective mutants, which are specifically blocked at the integration step. Results of IN-DNA photo-cross-linking, footprinting, and *in vitro* enzyme assays reveled other conserved catalytic core domain residues are likely to contact viral and target DNA during integration (Heuer and Brown 1997; Jenkins, Esposito et al. 1997; Esposito and Craigie 1998; Gerton, Ohgi et al. 1998; Heuer and Brown 1998; Appa, Shin et al. 2001; Dirac and Kjems 2001; Harper, Skinner et al. 2001). The conserved residues and structural features of the CCD $Phe^{139}$-$Gln^{146}$ flexible loop and abutting $Ser^{147}$-Val $^{165}$ alpha-helix are likely important for DNA binding during HIV-1 replication. Specifically, K159, Y143, and Q148 cross-linked to viral DNA substrates (Jenkins, Esposito et al. 1997; Esposito and Craigie 1998) and Q62, N120 likely contact viral and target DNA respectively during integration (van Gent, Groeneger et al. 1992; Esposito and Craigie 1998; Gerton, Ohgi et al. 1998; Chen, Wei et al. 1999). By analysising the roles of these residues in HIV-1 replication, studies showed that almost all singly substituted viral DNA-interacting mutants retained some capacity to replicate, which is different to active-site mutations wherein a single amino acid substitution is sufficient to render HIV-1 replication defective (Lu, Limon et al. 2005). An interesting finding is that mutations that reduced the binding of IN to viral cDNA, such as the N144Q, PYNP (position 142-145→KL), and KKK156, 159, 160AAA, resulted in severe impairment of virus infectivity, most likely by affecting the nuclear import of viral cDNA (Ikeda, Nishitsuji et al. 2004). For the C-terminal domain, experiments showed that IN 220-270 binds to both viral DNA as well as non-specific DNA, and two amino acid residues, arginine 262 and leucine 234, are critical to DNA binding (Lutzke, Vink et al. 1994; Lutzke and Plasterk 1998).

### I.4.3 Persistence and expression of unintegrated HIV-1 DNA

Reverse-transcribed linear viral cDNAs have two fates besides bona fide integration, none of which are precursors to integration: a. the conversion to 1 or 2-LTR circles by homologous or host cell nonhomologous DNA end-joint system (Farnet and Haseltine 1991); b. auto-integration to form defective, rearranged DNAs (Shoemaker, Hoffman et al. 1981; Brussel, Mathez et al. 2003). Recent analyses have concluded that circular forms of DNA are stable intracellularly, and undergo dilution only on cell division (Butler, Johnson et al. 2002; Pierson, Zhou et al. 2002; Brussel, Mathez et al. 2003; Bushman 2003). The persistence of unintegrated HIV-1 DNA in cells and its role are important but still unclear. Li *et al* suggested that the double-stranded ends of unintegrated linear viral cDNA mimic chromosomal breaks and may induce apoptosis (Lewis 1992). In order to avoid death, the cell may rapidly convert linear viral cDNA into circles, so class I IN mutant viruses generate higher levels of LTR circle when compared to the wild type (WT). In fact, large levels of circular forms of unintegrated HIV-1 DNA have been detected in the brains of some patients with AIDS dementia (Pang, Koyanagi et al. 1990). Also, unintegrated circular viral DNA, particularly 2-LTR circles, in the mononuclear cells of infected patients appears to be associated with high levels of plasma HIV-1 RNA, rapid decline in CD4 count, and clinical progression of AIDS (Panther, Coombs et al. 1998).

Although chromosomal integration is believed to be essential for HIV-1 life cycle (Panther, Coombs et al. 1998), evidence of HIV-1 gene expression from unintegrated DNA has accumulated for integration mutant viruses (Stevenson, Haggerty et al. 1990; Ansari-Lari, Donehower et al. 1995; Engelman and Craigie 1995; Wiskerchen and Muesing 1995; Wu and Marsh 2001; Poon and Chen 2003; Saenz, Loewen et al. 2004). Mutations of any of three residues that participate in the catalytic center (D64, D116, and E152) of HIV-1 IN produce phenotype that only affect integration steps (class I mutation). Cells infected with class I mutants contain higher levels of unintegrated DNA circles than do wild-

type infected cells, and class I HIV-1 IN mutants display 11.5 to 18% of WT activity in the multinuclear activation of galactosidase indicator (MAGI) assay (Ansari-Lari, Donehower et al. 1995; Engelman, Englund et al. 1995; Wiskerchen and Muesing 1995). Single-round replication of class I IN mutants carrying the luciferase gene (Luc) in the *nef* position produced 0.2 and 3.6%, respectively, of the level of WT luciferase in RD cells and HeLa cells (Masuda, Planelles et al. 1995; Poon and Chen 2003); in the latter case, the author demonstrated that HIV-1 Vpr can enhances expression from unintegrated HIV-1 DNA. More recently, some expressions of *tat* transcripts, *rev,* or the *rev* – dependent structural proteins, were found from unintegrated class I IN mutant HIV-1 DNA in resting T cells (Wu, Wakefield et al. 2001). Furthermore, Nakajiam *et al.* examined the infection properties of class I IN mutant HIV-1 in different T-cell lines and primary cells and found two cell lines (MT-4 and C8166) that showed spreading infections. MT4 cells even permitted serial passage of virus (Nakajima, Lu et al. 2001). Whether gene expression from unintegrated DNA plays a role in HIV-1 pathogenesis and the development of AIDS dementia *in vivo* is still an open question. Recent studies demonstrated that expression from unintegrated HIV-1 DNA has biological significance (Wu and Marsh 2001), and prevalent unintegrated HIV-1 DNA can be detected *in vivo* (Pang, Koyanagi et al. 1990; Chun, Carruth et al. 1997; Teo, Veryard et al. 1997).

### I.4.4 Characterization of class II IN mutants

IN is expressed as part of the Gag-Pol polyprotein precursor, which plays an important role in virion assembly and is essential for the formation of infectious virions. Mutagenesis of the C-terminal region of Pr160 $^{Gag\text{-}Pol}$ (IN domain) has been associated with defects in virion assembly, release, maturation, and protein composition (Shin, Taddeo et al. 1994; Ansari-Lari, Donehower et al. 1995; Engelman, Englund et al. 1995; Bukovsky and Gottlinger 1996; Quillent, Borman et al. 1996; Engelman, Liu et al. 1997). Moreover, these defective viruses are

also impaired in early steps of the virus life cycle, such as uncoating, viral DNA synthesis and nuclear import of PIC (Engelman, Englund et al. 1995; Masuda, Planelles et al. 1995; Cannon, Byles et al. 1996; Leavitt, Robles et al. 1996; Engelman, Liu et al. 1997; Gallay, Hope et al. 1997; Wu, Liu et al. 1999; Bouyac-Bertoia, Dvorin et al. 2001). These IN mutants are classified as Class II mutants. In contrast to class I IN mutants, which are specifically blocked at the integration step, the class II mutants cause pleiotropic defects at multiple stages of viral replication other than integration and such mutations may alter virus replication through various mechanisms in the viral life cycle.

Due to the pleiotropic nature of the class II IN mutants, assays to detect viral DNA synthesis, nuclear entry or integration have been developed and used to characterize the precise determinants in IN that cause each defect and understand a full function of IN.

For integration step, besides *in vitro* integration assay, the Alu-PCR, a nested PCR method with primers to the LTR and to repetitive Alu-elements found throughout the human genome can detect a bona fide integration *in vivo* (Chun, Stuyver et al. 1997). Since reduced integration can result from decreased reverse transcription or nuclear import, Alu-PCR assays are not always useful for dissecting the function of IN at the virus replication level. To directly analyze the integration function of IN, the Vpr-IN complementation assay can also be used to investigate the infectivity defects and catalytic activities of class I and class II IN mutants. Studies showed that the infectivity of integration-defective HIV-1 can be restored by incorporating WT IN or class II IN mutants, such as V165A, into assembling particles as Vpr-IN fusion protein (Fletcher, Soares et al. 1997; Wu, Liu et al. 1999), suggesting that these class II IN mutants do not specifically affect the integration step. Furthermore, analysis the cDNA synthesis and nuclear import profiles of replication-defective IN mutant viruses in cycling and nondividing cells are also essential for understanding the functional role of IN in HIV-1 replication.

Reverse transcription is a multiple step process requiring two template switches to generate full-length duplex cDNA (Fig.I.9). To analysis the cDNA synthesis, in addition to Southern blot, PCR primers can be designed to quantify specific steps in the sequential process (Julias, Ferris et al. 2001). Moreover, an RQ-PCR (Real-time Quantitative Polymerase Chain Reaction) assay with LTR- and gag-specific primers has been used to quantify levels of full-length and nearly full-length late reverse transcription products (Limon, Nakajima et al. 2002).

Nuclear entry of viral genomes is a prerequisite for viral integration. Detection of 2 LTR circle, is the most widely used assay for monitoring nuclear translocation of the PIC (Lewis 1992). Previous results established that class II IN mutant viruses were defective for two-LTR circle formation (Ansari-Lari, Donehower et al. 1995; Engelman, Englund et al. 1995; Leavitt, Robles et al. 1996; Engelman, Liu et al. 1997; Limon, Devroe et al. 2002). Although the defective nuclear import might be a phenotype common to all class II mutant (Limon, Devroe et al. 2002), other results indicated that reduced levels of reverse transcription might in large part account for the observed reductions in two-LTR circle formation (Leavitt, Robles et al. 1996). On the other hand, a higher level of two-LTR does not mean increased nuclear import. As previously reported, class I IN mutant, which have normal reverse transcription and PIC nuclear import, formed more 2-LTR circles than the WT virus by blocking the access of linear cDNAs to the integration end point (Engelman, Englund et al. 1995; Wiskerchen and Muesing 1995; Leavitt, Robles et al. 1996). Therefore, by using the methods described above, simultaneous analysis of the effect of IN on different steps of viral replication becomes possible.

**IN mutations influence the production of viral DNA**

Although reverse transcription is catalyzed by RT, and it can occur *in vitro* with recombinant RT, template, and primer, the process is more complex *in vivo*. In infected cells, reverse transcription takes place in the context of a nucleic acid-

protein complex that includes other viral and cellular factors. In addition to be affected by several viral factors, including MA, nucleocapsid, Nef, and Vif (Aiken and Trono 1995; Harrich, Ulich et al. 1997; Kiernan, Ono et al. 1998; Dettenhofer, Cen et al. 2000), the reverse transcription can also be influenced by IN (Tasara, Maga et al. 2001). Mutations in the HIV-1 IN coding sequence have been shown to impair viral DNA synthesis in infected cells. Deletion of entire IN (ΔIN) or a small portion (Δ22) of its C-terminus reduces the amount of early viral DNA products detected by PCR, and viruses containing either point mutations in the N-terminal zinc finger (H12L/A/C, H16V/A/C) or the central domain (F185A) exhibit a similar phenotype (Engelman, Englund et al. 1995; Masuda, Planelles et al. 1995; Engelman, Liu et al. 1997; Liu, Wu et al. 1999; Wu, Liu et al. 1999). Up to now, the mechanism by which IN mutations influence the production of viral DNA is not clear. Some studies demonstrated that a physical interaction exists between RT and IN of HIV-1 and MLV *in vitro* and this interaction is not mediated by nucleic acid bridging (Hu, Court et al. 1986; Wu, Liu et al. 1999; Tasara, Maga et al. 2001). Another report demonstrated that monoclonal antibodies generated against the minimal DNA binding domain in the C-terminus of IN block the interaction of recombinant IN and RT (Ishikawa, Okui et al. 1999). Recently, by using coimmunoprecipitation and GST pull down assays, two reports indicate that the C-terminal domain of IN is involved in interaction with RT, and C130S IN mutant virus abolished the ability of the virus to initiate reverse transcription presumably by disrupting the protein recognition interface of the C-terminal domain and abolishing its ability to interact with RT (Tan, Zhu et al. 2004; Zhu, Dobard et al. 2004).

**IN mutations influence the nuclear import of viral DNA**

There are three conserved regions, which were designated regions L, C, and N (FigI.10), located in the IN downstream of residue 179 in HIV-1 (Cannon, Byles et al. 1996). Region L is only found in the lentiviruses, while C and N are

conserved in all retroviruses. The region between sequence L and C contains a notable concentration of glutamine and basic residues in the lentiviruses and was designated region Q. Cannon *et al.* showed that IN mutant viruses within motif L (K186Q) and Q (Q214L, Q216L) abolished HIV-1 infectivity but did not affect particle production, morphology, reverse transcription, or nuclear import in T cell lines (Cannon, Byles et al. 1996). In 1997, Gallay first reported that IN recognized importin-α via a bipartite NLS, $NLS_P$ (KRK188) and $NLS_D$ (KELQKQITK219), in the L and Q regions since IN mutants K186Q and/or Q214L/Q216L resulted in the loss of interaction with karyopherin-α and exhibited a cytoplasmic localization (Gallay, Hope et al. 1997). However, the following studies on the subcellular localization of $NLS_P$ and $NLS_D$ mutant proteins were controversial. Although Petit *et al.* showed that alteration of L and Q sequence, either alone or in combination (K186Q; QQ214,6LL; K186Q/ QQ214,6L), resulted in the loss of nuclear accumulation of IN, other studies observed an effective nuclear localization of these mutant IN by using GFP-IN fusion proteins (Petit, Schwartz et al. 2000; Tsurutani, Kubo et al. 2000 ). In the context of viral infection, all studies confirmed the $NLS_P$ and $NLS_D$ mutant viruses were replication defective, but they failed to reveal evidence of altered nuclear localization in both dividing and non-dividing cells (Petit, Schwartz et al. 2000; Tsurutani, Kubo et al. 2000). Instead, the replication defect was suggested to reside at the levels of reverse transcription (Tsurutani, Kubo et al. 2000), integration (Petit, Schwartz et al. 2000) or postnuclear entry (Lu, Limon et al. 2004). However, a recent study by Lu *et al.* observed that infection of a Q region mutant (K215A/K219A) induced more than 3-fold lower luc activity compared to class I IN mutant D64N/D116N (Lu, Limon et al. 2004), suggesting there was a reduced level of viral DNA in the nucleus, which is accessible for *tat* and *nef* (Luc) expression. Moreover, their study revealed that, in the context of VSV-G pseudotyped virus infection in Jurkat cells, 2-LTR circle DNA levels of K215A/K219A and Q214L/Q216L were significantly lower than V165A and

C130G, even though the inhibition of viral reverse transcription mediated by these mutants was comparable (Lu, Limon et al. 2004).

For region C and N mutant viruses, most of them, such as K236E, L241A, L242A, K244A, E246K/A, K264E, R262A/R263A, R262A/K264A, K266A/E, were also replication defective and displayed reverse transcription defects and reductions of 2-LTR circles (Lu, Limon et al. 2004). The most plausible mutation is at W-235, which is conserved only in the lentivirus. While the W235A or W235E mutant has been shown to abolish viral replication, mutant W235F is replication competent (Engelman, Englund et al. 1995; Cannon, Byles et al. 1996; Leavitt, Robles et al. 1996). It is not clear where the block lies for W235A/E since the mutants have normal reverse transcription and nuclear import and *in vitro* integrase activity. It was suggested that the defect might occur during integration *in vivo* (Leavitt, Robles et al. 1996).

In 2001, the V165 and R166 residues in HIV-1 IN were shown to be critical for its NLS function (Bouyac-Bertoia, Dvorin et al. 2001). But reassessments of these V165/R166 functions by use of IN mutants V165A/R166A in several studies showed these mutants are class II IN mutant which are primarily defective in integration steps (Dvorin, Bell et al. 2002; Limon, Nakajima et al. 2002). Recently, mutants V165A and R166A were identified to be defective for binding to cellular protein LEDGF/p75 by His6-tag pull down assay (Cherepanov, Ambrosio et al. 2005). Interestingly, another IN mutant in the same region, Q168A, which also disrupted the interaction with LEDGF/p75, abolished the chromosomal targeting of IN without affecting its catalytic activity, resulting in integration and replication-deficient viruses. Furthermore, the mutation did not affect the nuclear import of HIV-1 IN (Emiliani, Mousnier et al. 2005).

Moreover, the effects of mutations at key residues for viral cDNA recognition (PYNP at positions 142 to 145, KKK156, 159,160) were evaluated in the context of viral replication and the biochemical properties of the recombinant protein (Ikeda, Nishitsuji et al. 2004). These studies indicated that mutations such

as N144Q, PYNP/KL or KKK156, 159,160/AAA, which reduced the binding of IN to viral cDNA, resulted in severe impairment of virus infectivity, most likely by affecting the nuclear import of viral DNA. IN residue C 130 also has been implicated in HIV-1 nuclear import through affecting IN multimerization more than functioning as an NLS *per se* (Petit, Schwartz et al. 2000). But following study reveals that HIV-1 C130G was defective for reverse transcription and is not essential for replication (Lu, Limon et al. 2004).

Taken together, until now, no IN mutant was clearly shown to principally block the nuclear import step. It appears that, by using typical viral infection system and analysis assays, it is difficult to draw a conclusion about which regions of IN contributes to nuclear import of HIV PIC due to the pleotropic effect of IN on viral replication. Hence, more studies are required in order to elucidate the exact role of IN in PIC nuclear import.

**IN mutations influence virion morphology, release, and/or assembly**

Defective viral particle assembly and release is one of the phenotypes associated with pleiotropic class II mutant (Bukovsky and Gottlinger 1996; Engelman 1999). Lack of IN can markedly affect HIV-1 particle production (Bukovsky, Song et al. 1999). Substituting Lys for Phe-185 dramatically increased the solubility of recombinant IN but affected particle assembly and release (Jenkins, Engelman et al. 1996; Engelman, Liu et al. 1997). HIV-1 IN/H12N, IN1-234, IN1-4 contained approximately 3-to 10-fold reductions in levels of RT and IN and also affected the appearance of virus particles, such as immature rings or aberrant mature particle (Engelman, Englund et al. 1995).

### I.4.5 Cellular proteins involved in HIV-1 integration

The integration of viral cDNA into the host chromosome is an essential step in the HIV-1 life cycle. The key protein for retroviral integration is IN.

However, a variety of cellular proteins have been put forward as important partners in the establishing the integrated provirus in the infected cells. Examples include the barrier-to-autointegration factor (BAF) (Lewis and Emerman 1994; Lee and Craigie 1998), high-mobility group protein A1 (HMGa1) (Farnet and Bushman 1997), integrase interactor 1(INI-1) (Kalpana, Marmon et al. 1994; Young 2001), DNA-PK (Daniel, Katz et al. 1999), DNA repair protein hRAD18 (Mulder, Chakrabarti et al. 2002), a cellular acetyltransferase p300 (Cereseto, Manganaro et al. 2005), and most recently, lens epithelium-derived growth factor/p75 (LEDGF/p75) (Busschots, Vercammen et al. 2005). Among them, BAF, INI-1 and LEDGF/p75 have been studied in detail as the co-factors of retroviral integration.

**The barrier-to-autointegration factor (BAF):** This factor is a small (10-kDa, 89 aa.) human protein, dimers of which bind directly but nonspecifically to double-stranded DNA (Cai, Huang et al. 1998; Zheng, Ghirlando et al. 2000; Lee, Haraguchi et al. 2001). The finding that lamina-associated polypeptide (LAP-2), a nuclear LEM (named for LAP2, emerin, and MAN1)-domain protein associated with lamina, interacts with BAF in a yeast two hybrid system suggests possible role of BAF in nuclear structure organization (Furukawa 1999). The potential role of this protein for retroviral integration still remains to be answered. BAF has been reported to protect M.MLV PIC against suicidal autointegration (Lee and Craigie 1998) or promote efficient intermolecular DNA recombination once a suitable chromosomal target site is located (Suzuki and Craigie 2002). *In vitro* integration assay indicated that recombinant human BAF protein restored both the integration activity of salt-disrupted HIV-1 PIC and the unique protein-DNA structure at the ends of HIV-1 by MM-PCR footprinting (Mu-mediated PCR footprinting) (Chen and Engelman 1998). Moreover, BAF was found present at low stoichiometry in HIV-1 virions and can bind directly to p55 Gag and MA (Mansharamani, Graham et al. 2003). However, the important role of BAF in

HIV-1 replication is questioned by a study that showed that BAF expression is very low or not detected in thymus and peripheral leukocytes, which are important target cells for HIV-1 infection (Mansharamani, Graham et al. 2003).

**Integrase interactor 1(INI1):** INI1/hSNF5 is one of the proteins that directly interact with IN and are incorporated into virion (Kalpana, Marmon et al. 1994; Morozov, Yung et al. 1998). It is a homologue of yeast transcription factor SNF5 and is a component of the ATP-dependent chromatin-remodeling mammalian SWI/SNF complex (Wang, Cote et al. 1996). This 385-amino-acid protein has three highly conserved regions including two direct imperfect repeats, repeat1 (Rpt1) and repeat 2(Rpt2), a C-terminal coiled-coil domain, and homology region (HR3). The Rpt1 region is necessary and sufficient to bind to HIV-1 IN (Morozov, Yung et al. 1998). The incorporation of INI1/hSNF5 into HIV-1 virions is directly correlated with its ability to exclusively interact with HIV-1 IN but not with other retroviral IN. At present it is unclear whether INI1 is really required for HIV-1 replication. However, studies demonstrated that INI1-deficient cells produced low amounts of virions that were poorly infectious, indicating that this protein is required for proper assembly of HIV-1 (Yung, Sorin et al. 2001). Recently, a fragment of IN1/hSNF5 (S6) (residues 183-294) spanning the minimal IN interaction domain was found to profoundly inhibit virus particle production of HIV-1 in a dominant negative manner (Yung, Sorin et al. 2004). Therefore, INI1 may play a role during the post-integration steps of HIV-1 replication.

**Lens epithelium derived growth factor (LEDGF/p75):** A novel cellular protein which directly interacts with HIV-1 IN and targets HIV-1 DNA integration is LEDGFp75. By using co-immunoprecipitation and yeast-two-hybrid analysis, LEDGF/p75 was identified as a binding partner of HIV-1 IN and the binding

region was located at its C-terminus (Cherepanov, Maertens et al. 2003; Turlure, Devroe et al. 2004; Emiliani, Mousnier et al. 2005).

The protein is predominantly localized in the nucleus, where it is associated with the chromosomes (Nishizawa, Usukura et al. 2001). The gene encoding LEDGF/p75 also encodes a smaller splice variant, p52, which shares a region of 325 residues with LEDGF/p75 at the N-terminus but contains eight additional amino acids (Ge, Si et al. 1998). Both proteins function as a survival factor and a transcriptional co-activator in the cell, but p52 fails to interact with HIV-1 IN *in vitro* or in living cells (Maertens, Cherepanov et al. 2003). LEDGF/p75 contains 530 amino acids and several functional domains. In the N-terminal region of it, a PWWP (for Pro-Trp-Trp-Pro) domain of 92 residues is present that functions as protein-protein interaction domain and/or DNA-binding domain (Stec, Nagl et al. 2000; Qiu, Sawada et al. 2002). A functional NLS ($^{146}$RRGRKRKAEKQ$^{156}$) was found by deletion mapping and site-directed mutagenesis (Maertens, Cherepanov et al. 2004; Vanegas, Llano et al. 2005). A conserved IN-binding domain (IBD) of 80 amino acids (residues 347-429) was mapped to the C-terminus (Cherepanov, Devroe et al. 2004). The structure of the IBD has been resolved by nuclear magnetic resonance (Cherepanov, Ambrosio et al. 2005) and is a compact right-handed bundle composed of five α helices. LEDGF/p75-binding site on IN was also studied and both the N-terminal zinc domain and the central core domain of IN were found to be involved in the interaction with LEDGF/p75 (Maertens, Cherepanov et al. 2003). Furthermore, Class II IN mutants V165A, R166A and L172A/K173A were most defective for binding to LEDGF/p75 by His6-tag pull down assay (Cherepanov, Ambrosio et al. 2005). In addition, *in vitro* integration assays with PIC showed that endogenous LEDGF/p75 is a component of HIV-1 and FIV PIC (Llano, Vanegas et al. 2004).

What is the functional role of LEDGF/p75 in HIV-1 replication? LEDGF/p75 has been shown to associate with HIV-1 IN in human cells (Cherepanov, Maertens et al. 2003). By using siRNA knock-down technique, initial studies suggested that endogenous LEDGF/p75 was both necessary and sufficient for accumulation of HIV-1 IN into the nucleus (Maertens, Cherepanov et al. 2003). Moreover, a single amino acid change in the NLS motif of LEDGF/p75 (K150A) was able to exclude the mutant LEDGF/p75 protein from the nucleus and abolish nuclear import of HIV-1 IN (Maertens, Cherepanov et al. 2004). Therefore, a possible role of LEDGF/p75 in nuclear import or, alternatively, in chromosomal tethering was initially proposed (Maertens, Cherepanov et al. 2003). However, in direct nuclear import assay, recombinant HIV-1 IN is still actively imported in the nucleus in the absence of LEDGF/p75 (Emiliani, Mousnier et al. 2005). More importantly, addition of a proteasome inhibitor to cells defective for LEDGF/p75 restores IN accumulation in the nucleus, suggesting that knock-down of LEDGF/p75 leads to a reduction of IN expression, likely resulting from proteasome activity, as LEDGF/p75 has been shown to increase the stability of HIV-1 IN in the cells by preventing proteasomal degradation (Llano, Delgado et al. 2004; Emiliani, Mousnier et al. 2005). Recently, in fluorescent correlation spectroscopy experiments, LEDGF/p75 was found to stimulate the binding of HIV-1 IN to DNA (Busschots, Vercammen et al. 2005). This *in vitro* result suggests that LEDGF/p75 more likely functions as a tethering factor for HIV IN to the chromosomes, which can explain the apparent nuclear accumulation of HIV-1 IN and association to mitotic chromosome. Moreover, Ciuffi et al found that LEDGF/p75 affects the choice of target sites for HIV-1 integration in cells (Ciuffi, Llano et al. 2005).

Mutagenesis was employed to elucidate the role of LEDGF/p75 in HIV-1 replication. An IN mutant Q168A was identified to abolish interaction with LEDGF/p75 through a random mutant IN library in a yeast-two-hybrid assay

(Emiliani, Mousnier et al. 2005). Although the Q168A recombinant IN displayed wild type IN activity *in vitro*, viruses containing IN Q168A were defective for replication due to a specific block at the integration step, whereas the nuclear import was not hampered. On the other hand, despite the mass of biochemical data implicating a role for LEDGF/p75 in HIV-1 replication, attempts to reduce the intracellular level of LEDGF/p75 protein using transient and stable siRNAs failed to ascribe a genetic link between endogenous levels of LEDGF/p75 protein and HIV-1 replication (Llano, Vanegas et al. 2004; Vandegraaff, Devroe et al. 2005). One explanation for why HIV-1 replicated in cells knocked-down for LEDGF/p75 expression is that relatively high residual levels of LEDGF/p75 protein within these cells might function to support normal levels of integration. On the other hand, with carefully controlled experiments, Vandekerckhove *et a.l* showed 3-5 fold decrease of HIV infection by transient or stable knockdown of LEDGF/p75 expression and confirmed the importance of LEDGF/p75 for HIV-1 replication (Vandekerckhove, Christ et al. 2006). All of these studies suggest that siRNA technology is limited and should be performed carefully in order to evaluate the role of cellular proteins in cell culture

### I.4.6 HIV-1 IN inhibitors (INIs)

Currently, there are three distinct mechanistic classes of drugs to combat HIV infection: inhibitors of the HIV-1 reverse transcritptase and protease enzymes and inhibitors of HIV entry. The therapeutic drug strategies such as HAART, that target the viral enzymes reverse transcriptase (RT) and protease (PR), have been successful in increasing patient lifespan and suppression of HIV-1 RNA plasma levels for a prolonged periods of time. However, viral replication is incompletely suppressed and drug resistance, patient adherence, and toxicity are serious challenges for HAART. On the other hand, viral entry is a complex process that can be divided into three steps: adsorption, co-receptor binding, and fusion. Each of these steps offers the potential for therapeutic intervention, and

several entry inhibitors are in preclinical or clinical trial (O'Hara and Olson 2002). From this broad class of potential inhibitors, one drug, enfuvirtide (ENF) or fuzeon, a fusion inhibitor, has gained approval from the FDA (Miller, Roller et al. 2004). But ENF is currently used as a salvage therapy owing to its prohibitive production cost and low oral bioavailability.

IN, the third of viral enzymes is the most promising of the new targets in preclinical or early clinical trials. IN is an essential part of the viral replication cycle. There is no mammalian homologue to this enzyme and the IN region of the *pol* gene is more conserved than either the RT or PR coding regions. All these features make IN an especially attractive drug target. Several new compounds that target specific integration steps have been identified and developed.

**Strand transfer inhibitors (STI)**: The STI binds to the IN portion of the viral IN-DNA complex within the enzyme's catalytic DDE in the context of the viral PIC after reverse transcription. However, the 3'-processing step can still occur and the STI bound PIC complex (STI-IN-viral DNA) enters the nucleus of the infected cell. Once in the nucleus, the presence of the STI blocks the IN enzymes's catalytic site and the STI-IN-viral DNA complex cannot bind to the cellular DNA (Hazuda, Felock et al. 2000). By random screening, compounds that contained a distinct β diketo acid motiety (DKAs: *4-aryl-2,4-diketobutanic acid*) were found to possesse a unique ability to specifically inhibit the DNA strand transfer step of integration (Hazuda, Blau et al. 1999; Hazuda, Felock et al. 2000; Pais, Zhang et al. 2002; Johnson, Marchand et al. 2004; Svarovskaia, Barr et al. 2004). STIs DKA derivatives, that are represented by naphthyridine carboxamide compounds L-870,810 and L-870,812, were developed. L-870,810 was active against viral strains exhibiting multidrug resistance to the currently licensed antiviral agents (Hazuda, Anthony et al. 2004). It was one of the first INIs to reach phase1 clinical trails, but the development of this drug was halted because

of toxicities observed in animals (Little S 2005). L-900,612 (MK-0518) is a new naphthyridine derivative and an upgraded compound from L-870,810. MK-0518 had promising early clinical trial results and has been advanced along the developmental pipeline entering phase 3 trials (Grinsztejn B 2006). MK-0518 had potent antiretroviral activity with 56% to 72% of patients achieving an HIV RNA level of less than 50 copies per milliliter at week 16 (Grinsztejn B 2006). Another STIs, GS-9137 ( *Dihydroquinoline-3-carboxylic* acid), is now in clinical phase 2 (Dejesus E 2006).

**3'-Processing Inhititor versus nuclear translocation inhibitor-** ***Styrylquinolines***: Styrylquinolines act both on 3'-processing and to a lesser extent strands transfer activities and FZ41 is the most potent compound from this group (Bonnenfant, Thomas et al. 2004). Tyrylquinolines have a unique mode of action, inhibiting the interaction between IN and cellular factors and competing for the LTR substrate (Zouhiri, Mouscadet et al. 2000; Bonnenfant, Thomas et al. 2004). Futhermore, Mousnier and colleagues demonstrated *in vitro* that FZ41 specifically and efficiently inhibited the nuclear import of IN in digitonin-permeabilized cells, which suggest that tyrylquinolines likely inhibit the interaction between IN and a cellular factor required for nuclear import of PIC (Mousnier, Leh et al. 2004). Tyrylquinolines class of compounds are very promising, however, better corroboration of the specific viral target in cell culture is needed.

**IN-DNA binding Inhibitors**: Among 5-H-pyrano-dipyrimidines compounds, V-165, which inhibited both HIV replication in cell culture and IN activity in enzymatic assays at micromolar concentrations, is the most potent IN-DNA binding inhititor (Pannecouque, Pluymers et al. 2002). V-165 prevent assembly of a stable complex between IN and viral DNA and consequently the next step of integration, 3'processing. V165 retained its activity when test against various drug-resistant HIV-1 strains (Pannecouque, Pluymers et al. 2002). Given the unique mode of action of pyrano-dipyrimidines and the lack of cross resistance to

DKAs, these agents may be good canadidates for further clinical development (Pannecouque, Pluymers et al. 2002; Fikkert, Van Maele et al. 2003; Witvrouw, Pannecouque et al. 2004).

**Other INIs**: There are a few other INIs in preclinical development, including host cell DNA repair protein inhibitor theophylline, nuclear import inhibitor ITI-367, antimicrobial peptide indolicidin, fungal polyketide mycelium integrasone and carbazole derivatives NIID.

# OBJECTIVES OF THIS STUDY

One of the features that distinguish HIV-1 and other lentiviruses from oncoretroviruses is their capacity to productively infect nondividing cells at the molecular level, the dichotomy between HIV-1 and oncoretroviruses has been attributed to the karyophilic properties of HIV PIC. The current model for HIV-1 nuclear entry is that the viral factors present in PICs contain nuclear localization signals that engage the cellular transport proteins, which direct the PIC through the nuclear pore. Several HIV-1 proteins, including MAp17, IN and Vpr, have been reported to have karyophilic properties and were shown to contribute to nuclear translocation of viral PICs (Bukrinsky, Haggerty et al. 1993; Heinzinger, Bukinsky et al. 1994; Connor, Chen et al. 1995; Gallay, Stitt et al. 1996; Gallay, Hope et al. 1997; Bukrinsky and Haffar 1998; Bouyac-Bertoia, Dvorin et al. 2001). In addition, a *cis*-acting element designated the central DNA flap, located in the 3' region of the *pol* gene sequence, was initially suggested to contribute to HIV-1 nuclear import in both dividing and nondividing cells (Follenzi, Ailles et al. 2000; Sirven, Pflumio et al. 2000; Zennou, Petit et al. 2000). However, several later reports have shown that the effects of the central DNA flap appear to be virus strain and host cell dependent (Dvorin, Bell et al. 2002; Limon, Nakajima et al. 2002). Therefore, the exact impact of the central DNA flap on HIV-1 infection is still an open question that remains to be clarified.

HIV-1 IN is a critical viral enzyme that catalyzes integration of viral cDNA into host chromosome. In addition, it has been demonstrated to have karayophilic property and was shown to be involved in the efficient nuclear import of HIV-1 PIC. However, up to now, the nuclear localization signals in IN, as well as the mechanism underlying IN nuclear import still remain to be determined. Two regions in IN ($^{186}$KRK and $^{211}$KELQKQITK) were initially proposed to constitute a bipartite NLS motif, since mutants K186Q and

Q214/216L lost the protein nuclear localization and their ability to bind to karyopherin α *in vitro* (Gallay, 1997). But following studies did not reveal the importance of these IN mutants on HIV-1 nuclear import (Petit, Schwartz et al. 2000; Tsurutani, Kubo et al. 2000; Lu, Limon et al. 2004). More recently, Fassati *et al* revealed that imp7 may contribute to HIV-1 PIC nuclear import using an *in vitro* nuclear import assay, and suggested that this cellular protein may interact with IN (Fassati, Gorlich et al. 2003). However, a recent study indicated that transient siRNA mediated knockdown of Imp7 had no effect on HIV-1 and SIV nuclear import in macrophages (Zielske and Stevenson 2005). Thus, more studies are required to define region(s) in IN that are essential for protein and PIC nuclear import and to elucidate the mechanism involved in the IN action for HIV nuclear import.

The goal of my study is to investigate the functional roles of the central DNA flap and IN during the early stage of HIV-1 replication, especially whether and how these factors contribute to the nuclear import of HIV DNA. The specific objectives include:

1. To investigate the precise impact of the central DNA flap on the HIV-1 infection and elucidate which step of viral replication is affected. This study includes two parts: 1) To generate a RT/IN trans-complement single-cycle replication system. This system will allow us to introduce mutations for disrupting DNA flap elements without affecting the enzymatic function of IN. 2) To investigate the importance of the central DNA flap for an efficient HIV-1 replication. The effect of DNA flap on early steps of HIV single-cycle replication, including reverse transcription, nuclear import and integration, will be analyzed by using specific PCR and southern blot assays.
2. To define the region(s) in IN that are essential for it nuclear localization as well as their contribution to HIV-1 PIC nuclear import and virus infection. 1) Mutagenic analyses, including deletion and substitution methods, will be used to investigate the contribution of different regions in HIV-1 IN to protein

nuclear localization. 2) To evaluate the impact of nuclear localization-defective IN mutants on HIV-1 single-cycle replication as well as their effects on viral DNA nuclear import.

3. To elucidate the molecular mechanism(s) involved in IN's action during HIV PIC nuclear import. This study will focus on the potential interaction of HIV-1 integrase (IN) with different cellular nuclear import receptors, including Imp7, by using a cell-based co-immunoprecipitation assay, and will also analyze the impact of this viral/cellular protein interaction on HIV replication

By using different approaches presented in this proposal, we hope to reach a more complete understanding of the actions of HIV-1 IN and the central DNA flap during the viral life cycle. Results from these experiments should provide valuable information for designing new and effective therapeutic approaches against HIV-1 before its integration.

# Chapter II

## Assessment of the Role of the Central DNA Flap in Human Immunodeficiency Virus Type 1 Replication using a Single-Cycle Replication System.

## ABSTRACT

Safe live-attenuated lentivirus capable of undergoing a single-round of replication constitutes a useful tool to study issues related to lentivirus dynamics and primary viral entry *in vivo* in animal models. As a proof of concept, we have tested and characterized a HIV-1 trans-complementation system consisting of RT and IN-defective HxBc2-derived proviral constructs and an expression plasmid encoding a Vpr-RT-IN fusion protein. In order to delineate the minimal *pol* gene sequence required in *cis* for efficient single-cycle replication, we performed a systematic deletion analysis of RT and IN gene sequences. Deletion of up to 2193 base pairs (bp) of *pol* gene sequence comprising of RT and 5' IN gene sequences was found to have no impact on HIV-1 gene expression and virus replication in this RT/IN trans-complemented single-cycle replication system. In contrast, maintenance of a 194 bp fragment corresponding to a region located at the 3'end of IN, which contains the central polypurine tract (cPPT) and the central termination sequence elements, was found to confer a 5- to 8-fold infectivity advantage to single-cycle replicating virus in a variety of cellular systems including MAGI cells and dividing and nondividing T cell lines. Introduction of mutations in the cPPT, which were reported to prevent the formation of the central DNA flap during reverse transcription, lead to a 5- to 7-fold decrease of viral production upon infection of human peripheral blood mononuclear cells (PBMCs). Furthermore, PCR analysis of reverse transcription, proviral DNA nuclear import and integration revealed that disruption of the cPPT affects by approximately seven-fold the level of late reverse transcribed products accumulating in the nucleus and as a consequence interferes with efficient proviral DNA integration. Overall, this study provides evidence that the central DNA flap enhances the establishment of HIV-1 infection in single-round replication assays primarily by facilitating proviral DNA nuclear import.

# INTRODUCTION

The complexity of human immunodeficiency virus (HIV-1) replication is attributed in a large part to the intricate interplay that takes place between *cis*-acting sequences present on viral nucleic acids and viral or host cell proteins that function *in trans*. The HIV-1 *pol* gene encodes three enzymatic proteins including protease (PR), reverse transcriptase (RT) and integrase (IN), which play critical roles during specific stages of the virus infection cycle. Soon after virus entry, RT catalyzes the conversion of the viral RNA genome into double-stranded proviral DNA while IN mediates proviral DNA integration into the host cell genome (for review, see (Coffin 1990; Golf 1992; Farnet and Bushman 1996; Greene and Peterlin 2002). Even though extensive *in vitro* biochemical and mechanistic studies have greatly contributed to a better understanding of the primary function and mode of action of RT and IN enzymes, studies performed in the context of HIV-1 infectious proviral clones have revealed that some mutations and/or internal deletions in RT or IN can significantly alter steps other than reverse transcription or integration such as virus assembly, release, or even inactivate virus infectivity (Ansari-Lari, Donehower et al. 1995; Engelman, Englund et al. 1995; Ansari-Lari and Gibbs 1996; Bukovsky and Gottlinger 1996; Gallay, Hope et al. 1997; Petit, Schwartz et al. 2000). These pleiotropic phenotypes resulting from mutagenic analysis suggest that RT and IN may play other roles, which are independent of their enzymatic activities. Furthermore, it cannot be excluded that introduction of mutations in RT and/or IN sequences may simultaneously affect *cis*-acting element(s) present within the *pol* gene sequence that are required for efficient virus replication. Several studies have reported that RT and/or IN enzymatic defects within HIV can be restored *in trans* either by expression of RT in newly-infected cells or through Vpr-mediated virion-incorporation of Vpr-RT-IN or Vpr-IN fusion proteins (Ansari-Lari and Gibbs 1996; Fletcher, Soares et al. 1997; Peng, Pan et al. 1997; Wu, Liu et al. 1997).

Interestingly, although this functional Vpr-RT-IN *trans*-complementation was shown to efficiently restore the infectivity of virus harboring premature stop codons in RT or IN open reading frames, trans-complementation of virus containing deletions in RT and/or IN sequences were found to reestablish viral infectivity very poorly (approximately 5 to 6 % of wild type virus infectivity levels) (Fletcher, Soares et al. 1997; Wu, Liu et al. 1997). These results suggest that other viral determinant(s) in *pol* gene sequence and/or synchronized expression and maturation of RT and/or IN from the Gag-Pol precursor polyprotein are required for efficient viral replication. Hence, assessing the requirement of HIV-1 *pol* gene sequence necessary for HIV-1 replication independently of enzymatic activities encoded by *pol* remains an important aim to fully understand the complexity of the HIV-1 replication cycle.

Retroviral replication requires the integration of reverse trancribed proviral DNA into the host cell genome. Prior to integration, viral reverse DNA transcripts must access the nucleus as part of a large ribonucleoprotein complex, named the preintegration complex (PIC). The lipid bylayer surrounding cellular nuclei contains numerous nuclear pore complexes (NPCs) that allow macromolecules of small diameter size (9 nm) and molecular weight (approximately between 40- to 60-kDa) to passively diffuse in and out of the nucleus (Nakielny and Dreyfuss 1999). The size of retroviral PICs is estimated to be roughly equivalent to the size of an eukaryotic ribosome such that they cannot be transported passively into the nucleus through NPCs (reviewed in (Greene and Peterlin 2002). Hence, oncoretrovirus, such of Moloney murine leukemia virus (MoMuLV), are believed to reach host cell chromosomes by timing proviral DNA nuclear transport with dissolution of the nuclear membrane that occurs at mitosis (Roe, Reynolds et al. 1993; Lewis and Emerman 1994). Indeed, MoMuLV productive infection requires cell to go through mitosis, although replication-defective MoMuLV mutants were recently identified as being blocked at the nuclear import step in dividing target cells (Yuan, Fassati et al. 2002). In contrast

to oncoretroviruses, HIV-1 and other lentiviruses do not depend on host cell mitosis to mediate the nuclear translocation of their PIC (Weinberg 1991; Lewis and Emerman 1994; Bukrinsky and Haffar 1998; de Noronha, Sherman et al. 2001; Le Rouzic, Mousnier et al. 2002; Maertens, Cherepanov et al. 2003). As a consequence, HIV-1 has the capacity to infect nondividing cell populations such as macrophages, mucosal dendritic cells, and nondividing T cells, which are believed to be critical for viral transmission, disease pathogenesis, and the establishment of persistent virus reservoirs. At the molecular level, HIV-1 PIC nuclear transport through intact NPCs was shown to occur by an active and energy-dependent process that involves the karyophilic properties of several PIC-associated viral proteins, including Matrix ($MAp17^{gag}$), IN and Vpr (Bukrinsky, Haggerty et al. 1993; Heinzinger, Bukinsky et al. 1994; Gallay, Hope et al. 1997; Fouchier, Meyer et al. 1998; Nie, Bergeron et al. 1998; Vodicka, Koepp et al. 1998; Haffar, Popov et al. 2000; Bouyac-Bertoia, Dvorin et al. 2001; de Noronha, Sherman et al. 2001). In addition, a *cis*-acting element named the central DNA flap was also shown to contribute to the nuclear import of HIV-1 proviral DNA in both dividing and nondividing cells (Zennou, Petit et al. 2000). The central DNA flap is a region of triple-stranded DNA created by two discrete half-genomic fragments with a central strand displacement event controlled in *cis* by a central polypurine tract (cPPT) and a central termination sequence (CTS) during HIV-1 reverse transcription (Charneau, Alizon et al. 1992; Charneau, Mirambeau et al. 1994). HIV-1 viruses carrying an inactivated cPPT or CTS were reported to exhibit a considerable impairment of viral replication in different dividing and nondividing target cells (Charneau, Alizon et al. 1992; Charneau, Mirambeau et al. 1994), presumably because of a defect at the level of the nuclear import of the PIC (Zennou, Petit et al. 2000). This observation raised the interesting possibility that retroviral PICs might interact with specific host factors to gain nuclear access even in cells that are actively dividing. However, these results have been put into questions recently by two studies (Dvorin, Bell et al. 2002; Limon, Nakajima et

al. 2002), which provided evidence indicating that the central DNA flap did not play a major role in either PIC nuclear import or HIV-1 replication in a variety of cell lines. Interestingly, in contrast to these studies that used replication-competent virus, numerous other studies have reported that the central DNA flap conferred an infection advantage of approximately 2 to 10 fold on single-round HIV-1-derived lentiviral vectors, thus suggesting that the central DNA flap facilitated early step(s) of lentiviral transduction (Parolin, Taddeo et al. 1996; Follenzi, Ailles et al. 2000; Sirven, Pflumio et al. 2000; Dardalhon, Herpers et al. 2001; Park and Kay 2001; Zennou, Serguera et al. 2001; Maele, Rijck et al. 2003). The exact impact of the central DNA flap on the early steps of HIV-1 infection is still an open question that remains to be clarified.

In this study, we took advantage of the ability of Vpr-RT-IN fusion proteins to trans-complement RT and IN proteins-defective HIV-1 viruses to delineate *pol* gene sequences, specifically RT and IN, that are important for HIV-1 replication in single-round replication assays. Our results reveal that the central DNA flap element confers a 5- to 8-fold infectious advantage to single-cycle replicating virus in a variety of cellular systems. We further investigated the impact of the central DNA flap on HIV-1 reverse transcription, nuclear import and integration by PCR analysis. Our results indicate that the central DNA flap enhances the establishment of HIV-1 infection in single-round replication assays by primarily facilitating nuclear import of proviral DNA.

## MATERIAL AND METHODS

**HIV-1 proviral constructs and plasmids.** The HIV-1 provirus plasmid HxBruR$^-$ used in this study is a chimera made between two closely related proviruses HxBc2 and BRU/LAI (Yao, Subbramanian et al. 1995). The phenotype of this molecular clone is 5' LTR *gag*$^+$ *pol*$^+$ *vif*$^+$ *vpr*$^-$ *tat*$^+$ *rev*$^+$ *vpu*$^-$ *env*$^+$ *nef*$^-$3' LTR (Yao, Subbramanian et al. 1995). The RT and IN-defective provirus R$^-$/RI$^-$ was constructed by replacing the first two amino acids of the RT reading frame of HxBruR$^-$ with two premature stop codons (TGATAG) using a two-steps polymerase chain reaction (PCR)-based method (Yao, Subbramanian et al. 1995). The 5' primer (5'-GCAGCTAGCAGGGAGACTAA-3') corresponds to a sequence located near the natural *Nhe*I site in *RT* (nucleotide (nt) position 3467, +1= start of BRU initiation of transcription) while the 3' primer (5'-CCTAATAAGGCCTTTCTTA-3') is derived from a sequence located near the *Stu*I site in *vif* (nt position 4987). Complementary oligonucleotide primers containing two stop codons (TGATAG) in place of the first two amino acids of the RT reading frame were used to generate a mutated *Nhe*I-*Stu*I PCR fragment. The resulting DNA fragment was then digested with *Nhe*I and *Stu*I and subcloned into an intermediate vector that contains an *Apa*I (nt position 1552)-*Sal*I (nt postion 5367) fragment derived from HxBru. Finally, the *Apa*I-*Sal*I fragment containing the two premature stop codons in RT was cloned back into the HxBruR$^-$ proviral construct. To construct IN gene-deleted proviruses R$^-$/RI$^-$ /ΔIN/flap+ and R$^-$/RI$^-$/ΔIN/flap$^-$, we first generated two PCR fragments using each of two 5'-oligos containing an engineered *Nhe*I site at nucleotide positions 4328 or 4522 (+1= start of BRU initiation of transcription) in IN gene sequence (5'-*Nhe*I-cPPT$^+$: 5'-ATCTTAAGCTAGCAGTACAATG-3'; 5'-*Nhe*I-cPPT$^-$: 5'-GGAAAGGGCTAGCAAAGCTCCT-3') and a 3'-oligo (5'-CAGGAGCTCAGTCTAGGATCTACTGGC-3') derived from sequence located

near nucleotide 5416 at the end of *vpr*. Both PCR fragments contain a natural *Sal*I site (nt position 5367) in *vpr*. Following digestion with *Sal*I, each of these PCR fragments were inserted into the $R^-/RI^-$ proviral plasmid which was first digested with *BspM*I (nt position 3904), treated with Klenow fragment of DNA polymerase I to fill the 5'end, and digested with *Sal*I restriction enzyme. The resulting proviral constructs $R^-/RI^-/\Delta IN/flap+$ and $R^-/RI^-/\Delta IN/flap^-$ carry respectively 416 and 610 bp deletions in the IN gene sequence. In the $R^-/RI^-/\Delta IN/flap^-$ provirus, the cPPT and CTS elements located in the 3' region of the IN gene were deleted (as shown in Fig. 1A). To generate $R^-$/RI-861, $R^-$/RI-1798, and $R^-/\Delta RI$ proviruses, *Apa*I-*Nhe*I PCR-amplified fragments were first generated using a 5'-oligo corresponding to nucleotide sequence located near the natural *Apa*I site (nt position 1552) in *gag* (5'-ATTGCAGGGCCCCTAGG-3') and a 3'-oligos derived from sequence located near nucleotide positions 2135, 2530 or 3467 in RT in which an engineered *Nhe*I site was introduced (5'-TCCGCTAGCTGGATCCACTGGTACAGTTTCAATAGG-3' 5'-GTTTACGCTAGCGATGGTAAATGCAG-3'; 5'-TAATCTAGACTCCCTGCTAGCTGCCCCAT-3'). Each PCR fragment was cloned into the $R^-/RI^-/\Delta IN/flap^+$ proviral vector using the natural *Apa*I site and the engineered *Nhe*I sites (at nt positions 4328, +1= start of BRU initiation of transcription) introduced 5' to the cPPT element. All proviral constructs were subsequently analyzed by DNA sequencing to confirm the presence of mutations or deletions.

To generate a plasmid expressing a Vpr-RT-IN fusion protein, we inserted a PCR-amplified Vpr cDNA into a SVCMV-in vector (Yao, Mouland et al. 1998) to generate SVCMV-in-R. This Vpr cDNA contains at the 3'end two additional codons encoding two glycine residues and a *Xba*I site that replaces the stop codon of *vpr*. A PCR-amplified RT and IN gene cDNA containing an engineered *Spe*I site in front of the RT reading frame and a natural *Stu*I site (nt

position 4987) after IN gene was digested with *Spe*I and *Stu*I and cloned in frame with Vpr into SVCMV-in-R plasmid. The resulting construct was named SVCMV-Vpr-RT-IN (shown in Fig. 1A).

**Cell lines, antibodies and chemicals.** Human embryonic kidney 293T and HeLa-CD4-LTR/-β-gal cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin and streptomycin. The human T-lymphoid MT4 and C8166 cells were maintained in RPMI-1640 medium. PBMCs were isolated from the blood of healthy adult volunteers by sedimentation in Ficoll-Hypaque (Sigma-Aldrich Canada Inc, Oakville, Ontario). Isolated PBMCs were stimulated with 0.1% phytohemaglutinin (PHA) and maintained in RPMI 1640 supplemented with 5% of IL-2. The HIV-1 positive human serum 162 and anti-HIVp24 monoclonal antibody used in this study were previously described (Yao, Subbramanian et al. 1995). 3'-Azido-3'deoxythymidine (AZT) and Aphidicolin, a DNA polymerase alpha inhibitor, were obtained from Sigma Inc.

**Transfection and the production of virus stocks.** DNA transfection in 293T cells was performed using the standard calcium phosphate DNA precipitation method. To produce virus stocks, supernatants from HIV-1 transfected 293T cells were collected at 48 h post-transfection and subjected to ultra-centrifugation (42,000 rpm for 1 hour at 4°C) to isolate virus. Quantification of virus stocks was determined by p24 measurements using an HIV-1 p24 ELISA Kit (purchased from the AIDS Vaccine Program of the Frederick Cancer Research and Development Center) or by RT activity assay (Yao, Mouland et al. 1998). To evaluate the infectivity of virus, equal amounts (15 ng of $p24^{gag}$ antigen/well) of virus were used to infect HeLa-CD4-β-Gal cells. The number of infected cells was evaluated by the MAGI assay 48 hours post-infection (p.i.), as described previously (Kimpton and Emerman 1992).

**Immunoprecipitation analyses.** Transfected 293T cells were starved in methionine-free DMEM for 30 min at 48 h post-transfection and then

metabolically labeled with 200 μCi of [$^{35}$S]methionine for 16 h. Labeled viruses were isolated, lysed and immunoprecipitated using anti-HIV-1 serum (162), as described previously (Yao, Subbramanian et al. 1995).

**Infection, [$^{3}$H]-thymidine uptake assay and cell cycle profile cytometry analysis**: To arrest C8166 T cells in G1 phase, 0.5x10$^{6}$ of cells/ml were cultured in the presence of aphidicolin (1.3μg/ml). At different time intervals, cell division was analyzed by a previously described [$^{3}$H]-thymidine uptake assay (Li, Simm et al. 1993). Cell cycle profile was also analyzed as previously described (Yao, Mouland et al. 1998).

To infect dividing and nondividing cells, C8166 T cells were pre-treated with 1.3 μg/ml of aphidicolin for 24 hours. Treated and non-treated cells were then infected with equivalent amounts of trans-complemented virus (65 ng p24$^{gag}$/10$^{6}$ cells) for 8 hours. Infected cells were washed and cultured in the absence or presence of the same concentration of aphidicolin. After 16 hours of infection, 10 μM of AZT was maintained in all cell cultures in order to compare all virus infection (including the replication-competent control virus) in a single-cycle replication manner. At 48 hours p.i., viral production in supernatants was evaluated by HIV-1 p24 ELISA.

To infect MT4 and human PBMCs, equivalent amounts of trans-complemented virus (65 or 135 ng of HIV-1 p24$^{gag}$/10$^{6}$ cells) were incubated with cells for 8 hours. At different time points, viral production levels were monitored by measurement of HIV-1 p24 antigen in each infected cell culture supernatant by HIV-1 p24 ELISA.

**PCR analysis of HIV-1 reverse transcription products and the integrated proviral DNA:** Human PBMCs (1x10$^{6}$) were infected with equal amounts of trans-complemented virus (135ng p24$^{gag}$/10$^{6}$cells) by spinoculation at 480 × *g* for 2 h. Cells were then washed three times and resuspended in RPMI containing 5% IL-2. At different time points after infection, equal number (2x10$^{6}$

cells) of PBMCs were collected, washed twice with PCR washing buffer (20mM Tris-HCl, pH8.0, 100mM KCl), and lysed in lysis buffer (PCR washing buffer containing 0.05% NP-40, 0.05% tween-20). Lysates were then incubated at 56°C for 30 min with proteinase K (100 μg/ml) and at 90°C for 10 min prior to phenol-chloroform DNA purification. To analyze viral DNA associated with the nucleus or the cytoplasm, a subcellular fractionation of infected PBMC was performed as described previously (Simon and Malim 1996). To quantify viral cDNA from each sample, all lysates were serially diluted 5-fold and subjected to PCR analysis. The primers used to detect late reverse transcription products were as follows: 5'-LTR-U3, 5'-GGATGGTGCTTCAAGCTAGTACC-3' (nt position 8807, +1= start of BRU of transcription initiation); 3'-Gag 5'-ACTGACGCTCTCGCACCCATCTCTCTC-3' (nt position 329). The probe for southern blot detection was generated by PCR with a 5'-LTR-U5 oligonucleotide, 5'-CTCTAGCAGTGGCGCCCGAACAGGGAC-3' (nt position 173) and the 3'-Gag oligo. PCR was carried out using 1x HotStar Taq Master Mix kit (QIAGEN, Mississauga, Ontario) and the program in which an initial heat activation step of 15 min at 95°C was followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 1 min and extension at 72°C for 1 min.

Integrated proviral DNA was detected in cell lysates by a modified nested Alu-PCR, in which following the first PCR, a second PCR was carried-out to amplify a portion of the HIV-1 LTR sequence from the first Alu-LTR PCR-amplified products. The first PCR was carried out using primers including 5'-Alu oligo (5'-TCCCAGCTACTCGGGAGGCTGAGG-3') and 3'-LTR oligo (5'-AGGCAAGCTTTATTGAGGGCTTAAGC-3') (nt position 9194) located respectively in the conserved region of human Alu sequence and in HIV-1 LTR. The primer used for both of the second nested PCR and for generating a probe are 5'-NI: 5'-CACACACAAGGCTACTTCCCT-3' and 3'-NI: 5'-GCCACTCCCCAGTCCCGCCC-3' (Chun, Stuyver et al. 1997).

To allow quantitative comparison of viral DNA contents in each reaction mixture, the $R^-$/ $RI^-$/ΔRI plasmid was used as standard for total HIV cDNA PCR detection. The plasmid was diluted in uninfected PBMC cellular DNA equivalent to the test samples. For detection of integrated viral DNA, each primer pair as described above was used in parallel to detect integrated viral DNA from serially diluted ACH-2 cells, which contain one viral copy/cell, in a background of uninfected PBMC cellular DNA. Detection of human β-2-adrenergic receptor (β2-AR) and β-globin genes was carried-out by PCR and used to estimate the DNA content of extracted chromosomal DNA preparations, as described previously (Simon and Malim 1996; Forget, Yao et al. 1998). The primers used for PCR are: 5'-β2-AR, 5'-TAGGCCTTCAAAAGAAGACCTGC-3'; 3'-β2-AR, 5'-CGTCTACTCCAGGGTCTTTCAG-3'; 5'-β-globin, 5'-CAACTTCATCCACGTTCACC-3'; 3'-β-globin, 5'-GAAGAGCCAAGGACAGGTAC-3'. Mitochondrial DNA was also amplified to standardize the cell-equivalent amounts of DNA extracted in cytoplasmic samples by using primers M1: 5'-GACGTTAGGTCAAGGTGTAG-3' and M2: 5'-GGTTGTCTGGTAGTAAGGTG-3'), which are described previously (Vandegraaff, Kumar et al. 2001).

All final PCR products were electrophoresed through 1.2% agarose gel and visualized by ethidium bromide staining or transferred to Zetaprobe nylon membrane (BioRad, Mississauga, Ont), subjected to Southern hybridization by using specific PCR DIG-Labeling probes (Roche Diagnostics, Laval, Que), and visualized by a chemiluminescent method. Densitometric analysis was performed using a Molecular Dynamics Personal densitometer and the Image Quant software version 3.22.

## RESULTS

### 1. Requirement of *pol* gene sequence for efficient HIV-1 single cycle infection.

The long-term objective of this study was to develop a single-cycle replication system to study issues related to lentivirus dynamics and primary viral entry *in vivo* in animal models. Initial feasibility studies were done using HIV-1 given the availability of basic reagents and molecular biology data. This single-cycle replication system consists of a replication-defective proviral construct carrying mutations or/and deletion in *pol* that can be complemented in *trans* with RT and IN using Vpr-mediated *trans*-incorporation, thus leading to viral particles that can undergo a single-round of replication. One important goal in designing and developing such a single-cycle replication system was of course to eliminate the possibility of recombination events that would generate replication-competent virions during viral production. It is therefore in the course of these studies that we initiated the characterization of minimal RT and IN gene sequences necessary and sufficient for maintaining efficient single-cycle replication.

First, we generated a RT and IN-defective HIV-1 provirus (R-/RI$^-$) with an intact *pol* gene sequence by replacing the first two amino acids of RT with two premature stop codons (TGA TAG) in a Vpr- and Nef-defective HxBc2-derived HIV-1 provirus (R$^-$) (Yao, Subbramanian et al. 1995). An expression plasmid encoding a Vpr-RT-IN fusion protein (CMV-R-RT-IN) was also made to trans-complement the RT and IN enzymatic activity defects (Fig.II.1A). Trans-complemented viruses were produced upon transfection of 293T cells with R$^-$/RI$^-$ and CMV-R-RT-IN plasmids. Consistent with a previous report (Wu, Liu et al. 1997) the RT and IN-defective R$^-$/RI$^-$ virus was found to be infectious in MAGI assay only when R$^-$/RI$^-$ virus were trans-complemented with RT and IN during viral production (FigII.1B, left panel). Maximum infectivity of trans-complemented R$^-$/RI$^-$ virus reached between 40 and 50 % of wild type level (R$^-$

virus) when the R⁻/RI⁻ proviral construct and the Vpr-RT-IN expression plasmid

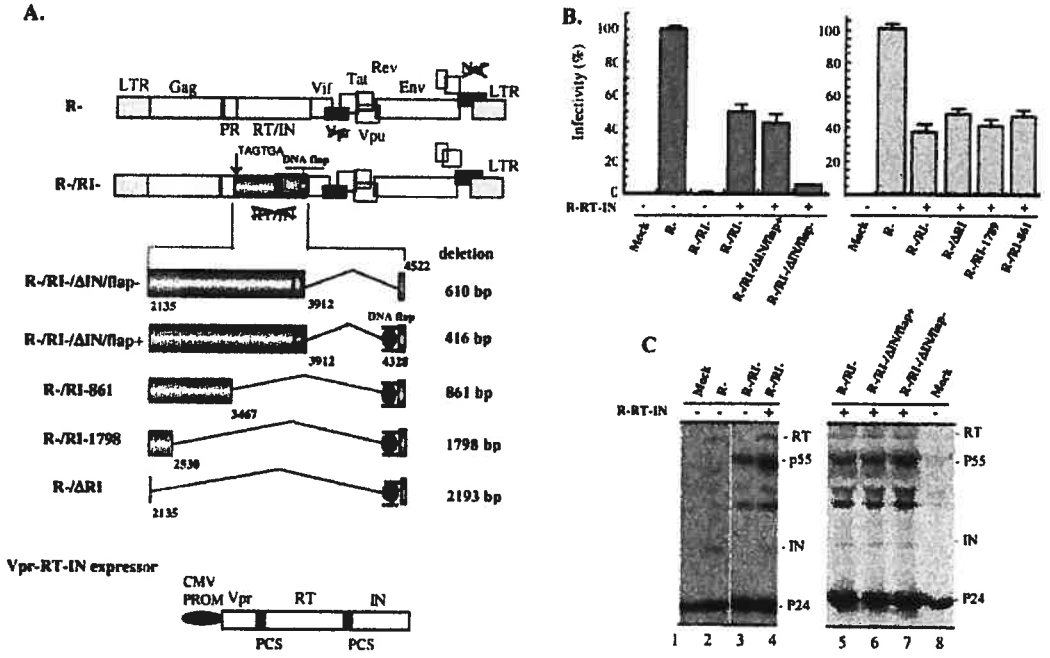


**Fig.II.1. Effect of HIV-1 IN and RT gene sequence on the infectivity of RT/IN trans-complemented virus. A).** Schematic structure of HIV-1 proviruses carrying mutation and deletions in the *pol* gene sequence and of the plasmid encoding the Vpr-RT-IN fusion protein. Provirus R⁻/RI⁻ was constructed by replacing the first two amino acid of RT with two premature stop codons (TGATAG) in HxBruR⁻ (R⁻) provirus. In R⁻/RI⁻/ΔIN/flap⁻ provirus, a 610 bp fragment of IN gene sequence (including cPPT/CTS) was deleted. The R⁻/RI⁻/ΔIN/flap⁺, R⁻/RI-861, R⁻/RI-1798, and R⁻/ΔRI proviruses carry different RT and/or IN gene sequences deletions but contain the194 bp sequence in the 3' end region of IN, which harbors the cPPT/CTS *cis*-acting elements. **B)** The infectivity of the trans-complemented virus produced in 293 T cells was evaluated by MAGI assay. **C).** To evaluate Vpr-mediated trans- incorporation of RT and IN in viral particles, radiolabeled virus were isolated from cell supernatants, lysed, immunoprecipitated with anti-HIV antibody and analyzed by 12.5% of SDS-PAGE.

were transfected at a molar ratio of 1:4. To test the efficiency of Vpr-mediated RT and IN trans-incorporation, transfected 293T cells were radiolabeled and the resulting viral particles analyzed for viral protein content by immunoprecipitation using anti-HIV-1 serum. Immunoprecipitation analysis revealed that, as expected,

the $R^-/RI^-$ viral particles did not contain any RT and IN proteins (Fig.II.1C, lane 3), whereas the trans-complemented $R^-/RI^-$ virus incorporated RT and IN proteins at levels comparable to the $R^-$ virus positive control, which expresses RT and IN in *cis* (Fig.II.1C, compare lane 4 to lane 2). Interestingly, it was noted that significant amounts of unprocessed $Pr55^{gag}$ accumulated in $R^-/RI^-$ viral particles (Fig.II.1C, compare lanes 3 and 4 to lane 2). This maturation defect is likely to result from impairment of protease activation given that the Gag-pol polyprotein precursor was truncated by early termination of RT and IN. This impairment of virus maturation was also found to be augmented in conditions where the $R^-/RI^-$ provirus was trans-complemented with the Vpr-RT-IN constructs presumably because of a saturation effect of protease due to over-expression of protease cleavage sites present in CMV-Vpr-RT-IN (Fig.II.1C, compare lanes 4 and 3). The 50 to 60% reduction of viral infectivity observed between trans-complemented $R^-/RI^-$ virus and the $R^-$ positive control is thus, likely a result of the viral maturation impairment (Fig.II.1B).

We next analyzed the requirement of RT and IN gene sequences for virus replication using this Vpr-RT-IN trans-complementation system. A series of RT and/or IN gene deleted proviruses derived from the $R^-/RI^-$ provirus were constructed (Fig.II. 1A). In the $R^-/RI^-/\Delta IN/flap^-$ provirus, a 610 bp sequence encompassing a large part of the IN gene and including the cPPT and CTS *cis*-acting sequences (from nt 3912 to 4522, +1 corresponds to the transcription initiation site of the BRU strain), which were previously shown to play an important role in HIV-1 replication, were deleted (Charneau, Alizon et al. 1992; Charneau, Mirambeau et al. 1994; Dardalhon, Herpers et al. 2001). In $R^-/RI^-/\Delta IN/flap^+$ provirus, a smaller deletion of 494 bp was introduced, thus leaving intact a 194 bp sequence at the 3' end of the IN gene. This 194 bp IN sequence contains the cPPT/CTS elements. To further test the impact of RT gene sequence

on virus replication, different regions of RT gene sequence were further deleted based on the $R^-/RI^-/\Delta IN/flap^+$ provirus and designated as $R^-$/RI-861, $R^-$/RI-1798, and $R^-/\Delta RI$ (Fig.II.1A). In the $R^-/\Delta RI$ provirus, all the RT and IN gene sequences, except the 194 bp containing the cPPT/CTS sequences, were deleted (deletion of 2193bp).

The infectivity of each trans-complemented RT and/or IN gene deleted virus, was analyzed by MAGI assay. Deletion of the 3' region of IN encompassing the cPPT/CTS elements resulted in a substantial decrease of viral infectivity, which varied from 5- to 7-fold as compared to the trans-complemented $R^-RI^-$ virus (Fig.II.1B, left panel). This sharp decrease of viral infectivity was not due to variation in the level of RT and/or IN trans-incorporated in viral particles since they were found to be similar in both trans-complemented viruses (Fig.II.1C, compare lane 7 and lane 5). In contrast, maintenance of a 196 bp in the 3' region of IN gene sequence, which includes the cPPT/CTS elements ($R^-/RI^-/\Delta IN/flap^+$) restored infectivity to a level similar to that of trans-complemented $R^-/RI^-$ virus (Fig.II.1B, left panel), thus confirming previous reports (Follenzi, Ailles et al. 2000; Dardalhon, Herpers et al. 2001; Zennou, Serguera et al. 2001; Maele, Rijck et al. 2003) indicating that the cPPT and CTS *cis*-acting elements confer an infectious advantage to lentiviral-based single-cycle replication systems. Interestingly, deletion of RT gene sequences had no impact on viral infectivity as long as the 194 bp sequence in the 3' end region of IN was intact. As shown in Fig.II.1B (right panel), the infectivity of trans-complemented $R^-$/RI-861, $R^-$/RI-1798 and $R^-/\Delta RI$ viruses was comparable to that of trans-complemented $R^-/RI^-$ virus. Analysis of RT and IN trans-incorporation levels in these RT and IN gene-deleted viruses did not reveal major differences as compared to the trans-complemented $R^-/RI^-$ virus (data not shown).

**2. Single-cycle replication of RT/IN trans-complemented virus in dividing and non-dividing CD4$^+$ T-cells.**

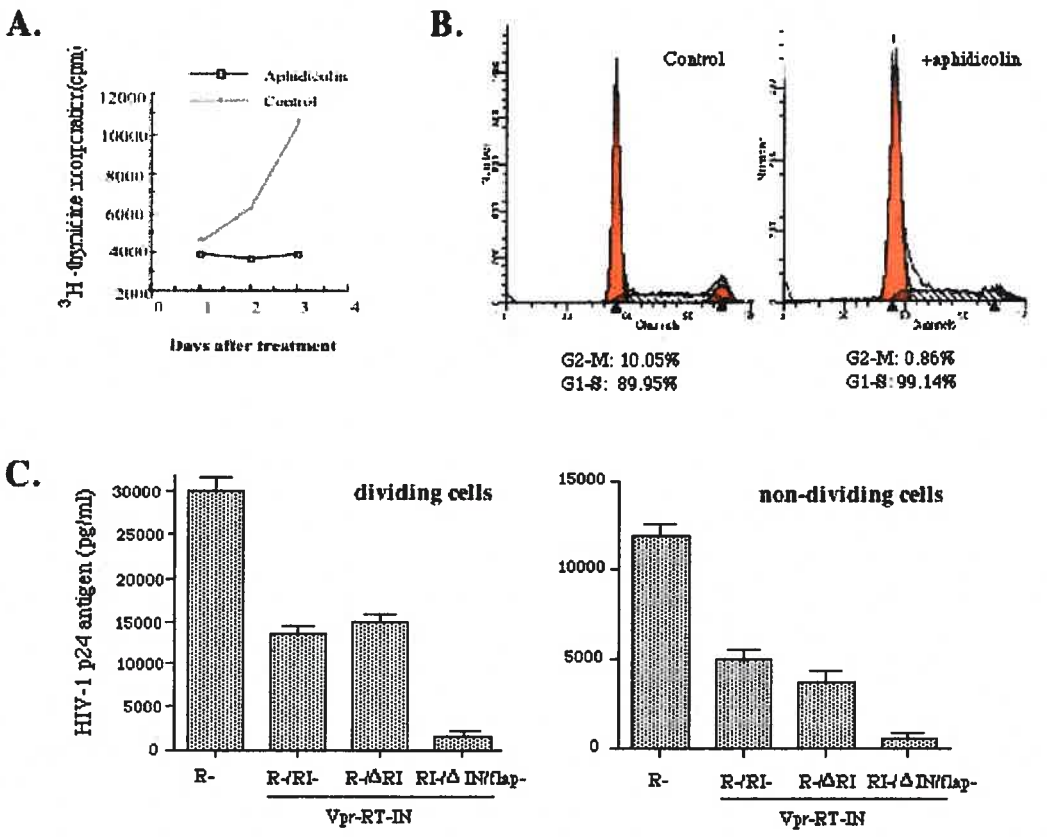


**Fig.II.2. Single cycle replication of trans-complemented RT/IN gene-deleted viruses in dividing and nondividing CD4$^+$ C8166 T cells.** **A)** To analyze aphidicolin-mediated blockage of cellular DNA synthesis, C8166 lymphocytes were incubated at $0{,}5x10^6$ cells/ml in the presence aphidicolin (1.3µg/ml) and [$^3$H]thymidine (1µCi/ml). At different time intervals, cells were lysed and the DNA-bound radioactivity was analyzed using a liquid scintillation counter. **B)** The cell cycle G1 arrest of aphidicolin-treated cells was confirmed by measurement of the cellular DNA content with FACScan. **C)** The aphidicolin-treated (right panel) or non-treated C8166 cells (left panel) were infected with equal amounts of the wild type and trans-complemented HIV-1 viruses. Viral production from each infected culture was monitored by measurement of HIV-1 p24$^{gag}$ antigen in the supernatants using p24 ELISA assay, 48 hours post infection. The results are representative of two independent experiments.

We next tested the single-cycle replication potential of the RT/IN trans-complemented viruses in the presence or absence of the 194 bp fragment encompassing the cPPT/CTS *cis*-acting element ($R^-/RI^-$, $R^-/\Delta RI$ and $R^-/RI^-/\Delta IN/flap^-$) in dividing and nondividing $CD4^+$ T-cells. Previous studies have shown that aphidicolin-growth arrested C8166 cells were susceptible to flap (Li, Simm et al. 1993; Zennou, Petit et al. 2000). To ensure that aphidicolin-treated C8166 cells were arrested at the G1 phase of the cell cycle, we measured [$^3$H]thymidine incorporation at different time intervals (Fig.II.2A) and analyzed the cell cycle profile at 18 h post-treatment (Fig.II.2B). Results of figure 2A and B confirm a complete blockage of cellular DNA synthesis and a G1 arrest of C8166 cells by aphidicolin treatment. The aphidicolin-treated (for 24 h) or non-treated C8166 cells were infected with equal amounts of trans-complemented virus. AZT (10 μM) was maintained in all cell cultures after 16 h of infection in order to compare virus infection during a single-cycle of replication. At 48h post infection (p.i.), supernatants were collected and viral production was monitored by measurement of virion-associated p24 antigen using HIV-1 p24 ELISA assay. The data from figure 2C reveal that wt, trans-complemented $R^-/RI^-$ and $R^-/\Delta RI$ viruses lead to a productive single-cycle infection in both dividing and nondividing C8166 cells. Consistent with the results obtained by MAGI assay (FigII.1B), infection with trans-complemented $R^-/RI^-$ and $R^-/\Delta RI$ viruses produced similar amounts of virus, yet virus production was two-fold lower relative to the levels detected with wild type ($R^-$) virus (Fig.II.2C). In contrast, the trans-complemented $R^-/RI^-/\Delta IN/flap^-$ virus replicated six- to seven-fold lower than $R^-/RI^-$ and $R^-/\Delta RI$ viruses in both dividing and non-dividing C8166 cells (Fig.II.2C). Overall, these experiments confirm that the 3' region of the IN gene sequence harbors *cis*-acting determinants(s) that enhance substantially the replication of HIV-1 toward dividing and nondividing CD4+ T cells in the context of single-

cycle infection system, while other RT and IN gene sequences are clearly dispensable.

**3. The cPPT contributes to efficient single-cycle replication of trans-complemented RT/IN gene deleted virus.**

To further confirm that *cis*-acting element(s) in the 3' region of IN gene sequence contribute to efficient single-cycle replication, we introduced a 10 bp substitution in the cPPT element in the R⁻/ΔRI provirus and generated a cPPT-defective mutant designated R⁻/ΔRI/cPPT⁻ (Fig.II.3A). These specific mutations in the cPPT element have previously been reported to prevent the formation of the central DNA flap during reverse transcription (Zennou, Petit et al. 2000; Dvorin, Bell et al. 2002; Limon, Nakajima et al. 2002). Prior to testing the infectivity of trans-complemented R⁻/ΔRI/cPPT⁻ virus, the levels of trans-incorporated RT and IN was examined by radiolabeling and immunoprecipitation in both the cPPT mutant and control viruses as described in figure II.1. Similar levels of virion-associated RT, IN, $p24^{gag}$ and $p55^{gag}$ were detected in trans-complemented R⁻/RI⁻, R⁻/ΔRI and R⁻/ΔRI/cPPT⁻ virus preparations (Fig.II.3B), suggesting that the cPPT mutations had no significant effect on virus assembly and RT and IN trans-incorporation. To compare the replication potential of trans-complemented R⁻/ΔRI/cPPT⁻ and R⁻/ΔRI viruses, $CD4^+$ MT4 cells and PHA-stimulated human PBMC (h-PBMC) were infected with equal amounts of each virus stock for 8 hours and, at different time intervals, virion-associated $p24^{gag}$ antigen levels in the supernatant were measured by anti-p24 ELISA. Disruption of the cPPT was found to decrease substantially viral replication in both MT4 T cells and activated PBMC. Cells infected with the trans-complemented R⁻/ΔRI/cPPT⁻ viruses were found to produce 5- to 7-fold fewer viruses than cells infected with the trans-complemented R⁻/ΔRI control virus (Fig. II.3C and D). Interestingly, this reduction in viral replication observed with the cPPT mutant (R⁻/ΔRI/cPPT⁻) was

comparable to that obtained with the IN gene deletion mutant ($R^-/RI^-/\Delta IN/flap^-$) (Fig.II.2). Hence, we conclude that the central DNA flap is the necessary determinant in the 3' region of IN gene sequence that contributes to efficient single-cycle replication of trans-complemented virus.

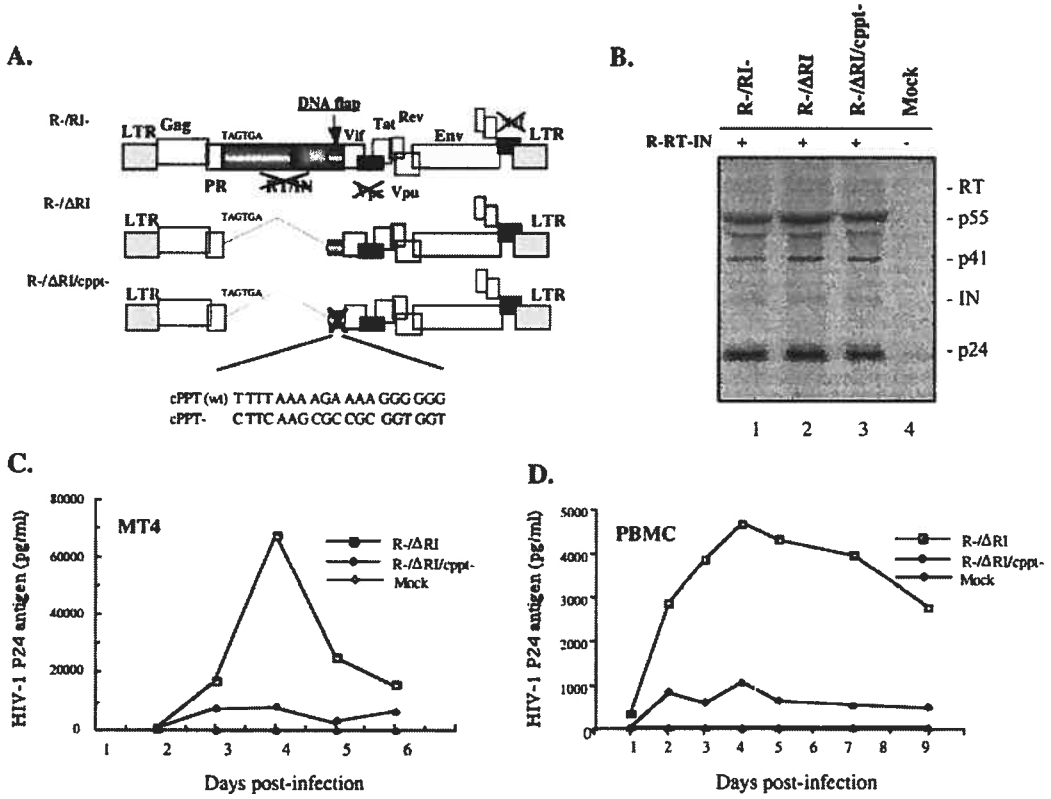


Fig.II.3. **The central DNA flap contributes to efficient single-cycle replication of trans-complemented RT/IN gene-deleted virus.** A). Schematic structure of RT/IN gene-deleted HIV-1 provirus ($R^-/\Delta RI$) and cPPT$^-$ mutant ($R^-/\Delta RI/cPPT^-$). The cPPT element was inactivated by introduction of ten nucleotide substitution mutations, as indicated. B) To compare trans-incorporation of RT and IN, $^{35}$S-methionine-radiolabeled virus were collected from different co-transfected 293T cells, lysed and analyzed with anti-HIV immunoprecipitation. To test the replication potential of each virus stock, CD4+ MT4 T cells C) or PHA-stimulated human PBMCs D) were infected with equal amounts of $R^-/\Delta RI$ or $R^-/\Delta RI/cPPT^-$ viruses. At different time intervals after infection, viral production was monitored by measurement of HIV-1 $p24^{gag}$ antigen in the supernatants with p24 ELISA assay. Results are representative of two independent experiments.

**4. Effect of the central DNA flap on HIV-1 proviral DNA integration in h-PBMC.**

To investigate the mechanism(s) underlying the action of the central DNA flap during single-cycle replication, we first analyzed the efficiency of proviral DNA integration in h-PBMC infected with $cPPT^+$ or $cPPT^-$ trans-complemented

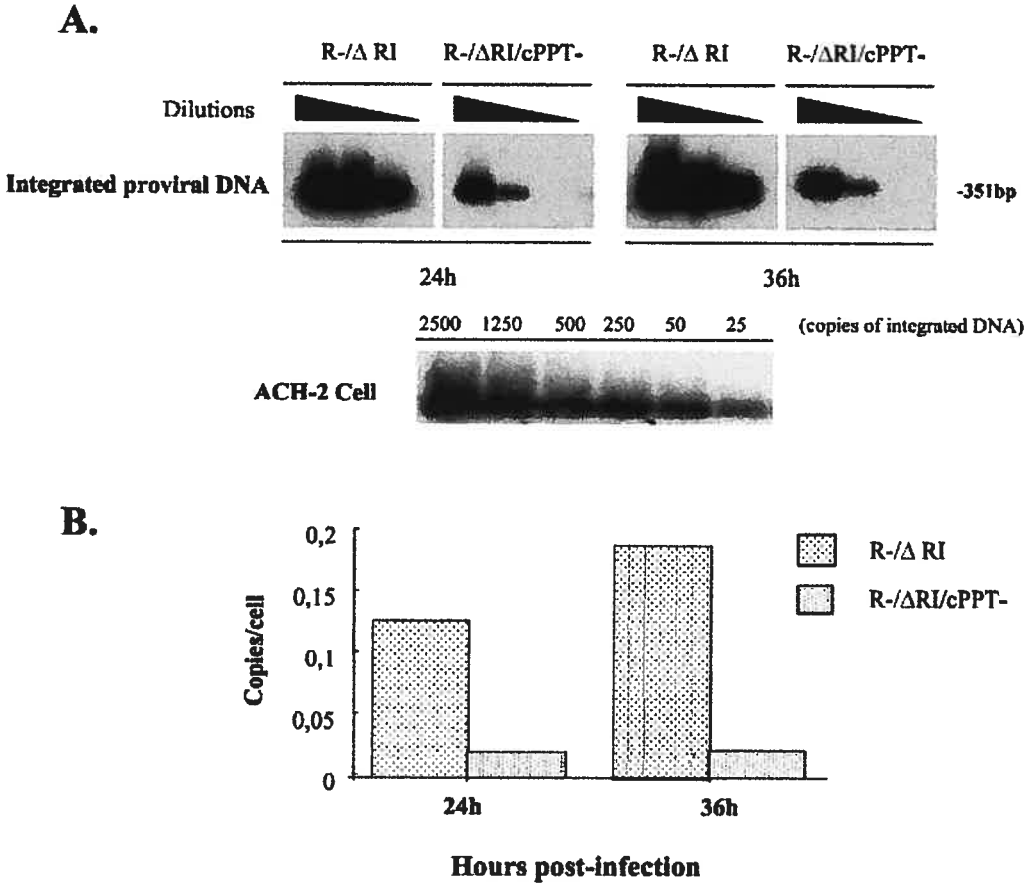


**Fig.II.4. Effect of the central DNA flap on HIV-1 proviral DNA integration in human PBMC. A).** Human PBMCs were infected with $R^-/\Delta RI$ or $R^-/\Delta RI/cPPT^-$ virus (125 ng $p24/10^6$ cells). At 24 and 36 hours p.i., cells were lysed and serial-diluted cell lysates were analyzed by two-step Alu-PCR and Southern blot for specific detection of integrated proviral DNA from infected PBMC (Upper panel) or the ACH-2 cells as quantitative control (lower panel). **B).** Quantitative analysis of integrated proviral DNA in single-cycle infection. Bands in panel A were quantified by laser densitometry and the number of integrated

proviral DNA copy/cell was determined using the PCR-generated standard curve of ACH-2 cells.

viruses using a previously described sensitive Alu-PCR technique (Chun, Stuyver et al. 1997). Results reveal that the levels of integrated proviral DNA detected in the $R^-/\Delta RI/cPPT^-$ sample were five to seven-fold lower than those detected in the $R^-/\Delta RI$ sample at both 24 and 36 hours p.i. (Fig.II.4A and B), indicating that disruption of the central DNA flap reduces significantly proviral DNA integration during single-round HIV-1 replication. Interestingly, this significant decrease of proviral DNA integration correlated well with the 5- to 8-fold replication defect observed with the cPPT-defective virus, indicating that the central DNA flap contributes to efficient viral single cycle replication by acting on early stage(s) of viral replication at and/or prior to viral integration.

**5. Effect of central DNA flap on late reverse transcribed DNA products and viral cDNA nuclear import in h-PBMC.**

To further investigate at which early step(s) of the infection cycle the central DNA flap acts, we analyzed total amounts of viral cDNA present at different early time points following infection of h-PBMC with equal amounts of $R^-/\Delta RI$ or $R^-/\Delta RI/cPPT^-$ viruses. As shown in figure II.5A, at 6 hours p.i., similar amounts of total viral cDNA were detected in $R^-/\Delta RI$ and $R^-/\Delta RI/cPPT^-$ infected cells (4.9 copies/cell versus 4.6 viral copies/cell) (Fig.II.5B), suggesting that both trans-complemented virus entered cells with similar efficiencies and underwent uncoating and reverse transcription with comparable rates. In contrast, between 6 and 24 hours p.i., total amounts of late $cPPT^-$ reverse transcribed products decreased at a rate that was clearly different from viral $cPPT^+$ cDNA products. At 12 h and 24 h p.i, levels of viral $cPPT^-$ cDNA were reduced by approximately 45% and 40% as compared to the levels of $cPPT^+$ cDNA, which stayed quite stable during the same time interval (approximately between 87% and 88% of their levels at 6 hours) (Fig.II.5A and B). At later time points (between 24h and

48h), boh cPPT$^+$ and cPPT$^-$ viral cDNAs decreased at similar rates most probably as a result of the dilution of unintegrated viral cDNA that occurs upon cell division. This difference in the rate of viral cDNA decrease detected between R$^-$/ΔRI and R$^-$/ΔRI/cPPT$^-$ infection was not due to intrinsic variation between samples since similar levels of a control cellular DNA (β2-AR gene) was detected by PCR in each sample (Fig.II.5A, lower panel). These results indicate that the central DNA flap does not interfere with the rate of reverse transcription step per se but appears to influence the rate of accumulation of total viral cDNA product.

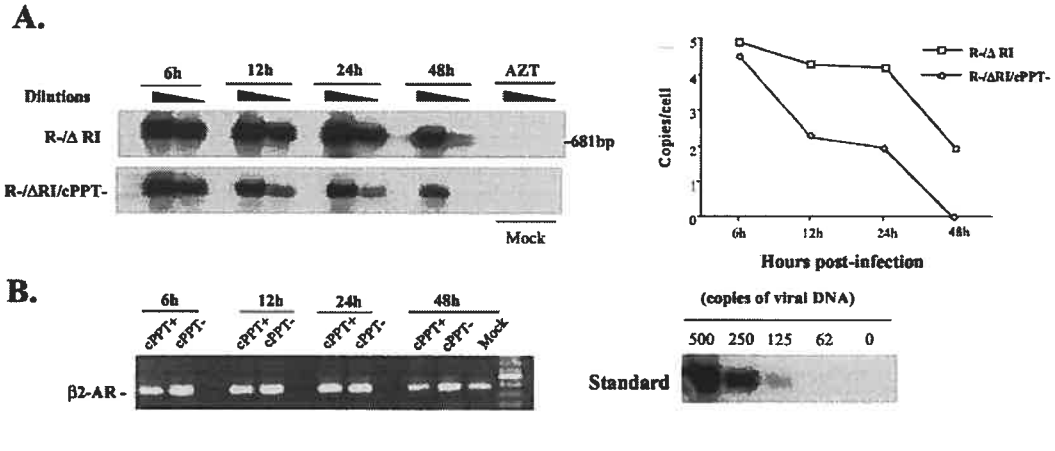


**Fig.II.5. Effect of the central DNA flap on the steady state levels of HIV-1 reverse transcription products. A).** Human PBMCs were infected with trans-complemented R$^-$/ΔRI and R$^-$/ΔRI/cPPT$^-$ viruses (125 ng of p24/$10^6$ cells) for 2 hours., the AZT (10 μM) pre-treated PBMCs were used as negative control. At each indicated time point, the total DNA was extracted and then, serial dilutions of extracted DNA were analyzed for late reverse transcription products by PCR using LTR-Gag primers and with Southern blot(A, left). HIV-1 late reverse transcription products detected in the left panel were quantified by laser densitometry. The diagram at the right shows the number of HIV-1 cDNA copies per cell as determined using the PCR-generated standard curve (A, right). These results are representative of those obtained in two experiments. **B).** Serial diluted R-/ΔRI plasmid DNA was used as a standard for DNA copy quantification (lower right panel). To monitor cellular DNA levels in each sample, the cellular β2-AR staining (lower left panel).

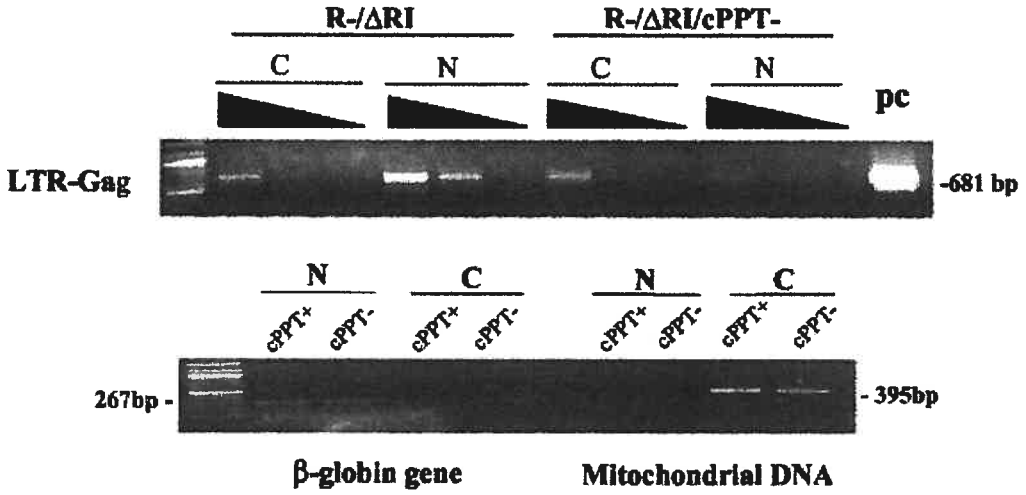


**Fig.II.6. The central DNA flap facilitates viral cDNA nuclear import. Upper panel:** Human PBMCs were infected with identical amounts of $R^-/\Delta RI$ and $R^-/\Delta RI/cPPT^-$ viruses. At 24 hours p.i., $2x10^6$ cells were fractionated into cytoplasmic and nuclear fractions as described in Materials and Methods. The amounts of viral DNA in the cytoplasmic and nuclear fractions were analyzed by PCR using HIV-1 LTR-Gag primers and visualized by ethidium bromide staining. Meanwhile the $R^-/\Delta RI$ plasmid DNA was used as a template dung PCR as positive control (pc). **Lower panel:** Purity and DNA content of each subcellular fraction were monitored by PCR detection of human globin gene and mitochondrial DNA and visualized by ethidium bromide staining (N. Nuclear fraction; C. cytoplasmic fraction). gene was amplified by PCR and visualized by ethidium bromide

In parallel, we analyzed viral cDNA nuclear import by subcellular fractionation and subsequent detection of viral cDNA associated with nuclear or cytoplasmic fractions as previously described (Simon and Malim 1996). Human PBMCs were infected with equivalent amounts of trans-complemented $R^-/\Delta RI/cPPT^-$ or $R^-/\Delta RI$ viruses and cytoplasmic and nuclear fractions were isolated from the same number of cells at 24 hours p.i. All fractions were then analyzed by PCR and the presence of total viral DNA was visualized by ethidium bromide staining. Results of figure II.6A reveal that at 24 h p.i. Total amounts of viral cDNA in the $R^-/\Delta RI$ infected sample (including cytoplasmic and nuclear

fractions) were approximately three-fold higher than those detected in the $R^-$ /ΔRI/cPPT$^-$ infected sample, thus confirming the data obtained in figure II.5. Interestingly, while approximately 75% of total viral cDNA was detected in the nuclear fraction of $R^-$/ΔRI infected cells; only 30% of total viral cDNA was detected in the nucleus of $R^-$/ΔRI/cPPT$^-$ infected cells (Fig.II.6,upper panel). Moreover, the absolute levels of nuclear associated viral cDNA levels were approximately seven-fold higher with the wild type virus than with the cPPT-defective virus. The integrity of the fractionation procedure was validated by detection of mitochondrial DNA and β-globin DNA, which were found solely in the cytoplasm and the nucleus respectively (Fig.II.6, lower panel). In addition, levels of mitochondrial and β-globin DNAs were similar in both $R^-$/ΔRI and $R^-$ /ΔRI/cPPT$^-$ subcellular fractions confirming that equivalent amounts of nuclear and cyctoplasmic fractions were analyzed. Overall, these results indicate that the central DNA flap influences the transport and the accumulation of late reverse transcribed products in the nucleus during single-cycle infection.

## DISCUSSION

In this study, we have investigated the impact of HIV-1 RT and IN gene sequences on HIV-1 replication independently of RT and IN functions using a RT and IN trans-complemented single-cycle replication system. Our results reveal that a 194 bp sequence located at the 3'end of IN gene is necessary for efficient HIV-1 single-cycle replication in dividing and nondividing T cells, while a deletion of up to 2193 bp of RT and 5' IN gene sequence has minimal impact on HIV-1 gene expression and virus replication in this system. Mutagenic analysis further indicate that mutations in this critical 3' region of IN gene sequence, which target the central DNA flap element cPPT, interfere with single-round replication in T cells and h-PBMCs, indicating that the central DNA flap constitutes an important determinant for efficient HIV-1 replication. In an

attempt to assess the role of the central DNA flap in this single-cycle replication system, we systematically analyzed by PCR the reverse transcription, proviral DNA nuclear import and integration steps of the infection cycle. Our data reveal that disruption of the central DNA flap interferes with the transport and the accumulation of total reverse transcribed DNA products in the nucleus.

Establishment of HIV-1 infection in dividing and nondividing cells depends on the ability of the viral PIC to actively translocate into the nucleus and to integrate into host chromosomes. Several viral proteins including Matrix ($MAp17^{gag}$), IN and Vpr have been shown to contribute to efficient viral PICs nuclear import in nondividing cells by interacting with components of the host cell nuclear import machinery via putative nuclear localization signals found within these viral proteins (Bukrinsky, Haggerty et al. 1993; Heinzinger, Bukinsky et al. 1994; Gallay, Hope et al. 1997; Vodicka, Koepp et al. 1998; Haffar, Popov et al. 2000; Bouyac-Bertoia, Dvorin et al. 2001). In addition, it hasbeen reported that formation of the central DNA flap is necessary for HIV-1 replication in both dividing and nondividing cells presumably because this *cis*-acting sequence acts as a determinant of HIV-1 PIC nuclear import (Zennou, Petit et al. 2000). However, this important role of the central DNA flap in HIV-1 replication and nuclear import has been questioned by two recent studies (Dvorin, Bell et al. 2002; Limon, Nakajima et al. 2002), which compared the infectivity and nuclear localization phenotypes of replication-competent wild type and flap-defective viruses and found that the central DNA flap did not play a major role in either PIC nuclear import or HIV-1 replication in a variety of cell lines. Although these studies did not find that the central DNA flap played a significant role in the viral life cycle in immortalized cell lines, they still observed a modest replication delay in PBMCs. In contrast, using a single-cycle infection system, we found that the central DNA flap indeed contributes to efficient single-round replication of RT/IN trans-complemented virus in different cell types including MAGI cells,

MT4 and C8166 T cell lines as well as in h-PBMCs (Fig.II.1-3). RT-IN trans-complemented virus harboring mutations that were reported to prevent the formation of the central DNA flap displayed a level of replication that was decreased by 5- to 8-fold as compared to trans-complemented virus containing an intact central DNA flap (Fig.II.2 and 3). These results differ from previously published work using replication-competent virus where the requirement for the DNA flap was found to be either absolute (Zennou, Petit et al. 2000) or marginal (Dvorin, Bell et al. 2002; Limon, Nakajima et al. 2002) but are consistent with the findings reported by several previous studies that showed that the central DNA flap conferred an infection advantage of 2- to 10-fold on VSV-G pseudotyped single-round HIV-1 vectors (Parolin, Taddeo et al. 1996; Follenzi, Ailles et al. 2000; Sirven, Pflumio et al. 2000; Dardalhon, Herpers et al. 2001; Park and Kay 2001; Zennou, Serguera et al. 2001; Maele, Rijck et al. 2003). It was suggested by Zennou *et al.* (Zennou, Petit et al. 2000) that the central DNA flap was not absolutely essential in single-round HIV-1 vectors because the smaller size of the vector genomes precluded the absolute requirement for the DNA flap during nuclear import. Clearly, in the case of our replication system, the lack of an essential role of the central DNA in viral replication cannot be attributed to the size of the viral genome given that our central DNA flap-defective proviral constructs were comparable in size to wild type virus and still replicated albeit at low levels (Fig.II.2 and 3). On the basis of our results, we conclude that the central DNA flap is not essential for HIV-1 replication but rather has an enhancing effect on HIV-1 single-round replication in both dividing and nondividing cells. However, it is still unclear why this substantial defect in single-round infectivity caused by disruption of the DNA flap does not translate into a detectable difference when replication kinetic is monitored using replication competent virus as shown recently (Dvorin, Bell et al. 2002; Limon, Nakajima et al. 2002). Clearly, more studies in this area are required to understand this discrepancy.

In an attempt to understand the mechanism(s) underlying the effect of the central DNA flap during HIV-1 single cycle replication, we analyzed by PCR the amount of integrated proviral DNA in the presence or absence of the central DNA flap. Our results clearly show that disruption of the DNA flap results in a 5- to 7-fold decrease in proviral DNA integration (Fig.II.4). This significant defect of proviral DNA integration correlates well with the decreased single-cycle replication potential (5-to 8-fold) observed with trans-complemented flap-defective virus. Hence, it is clear that the central DNA flap contributes to efficient single-cycle viral replication by acting on early stage(s) of HIV-1 infection cycle at and/or prior to viral integration. To further understand this effect of the central DNA flap, we analyzed the total late reverse transcribed DNA products over time during $cPPT^+$ or $cPPT^-$ single-cycle viral infection and determined the distribution of total viral cDNA in the nucleus and the cytoplasm. Quantification of total reverse transcribed viral cDNA indicates that the central DNA flap does not significantly influence the amount of viral transcripts produced at early time points (6 hours) but contributes to maintenance of high level (approximately 2-3 fold) of viral cDNA up to 24 hours p.i., as compared to flap-defective virus (Fig.II.5 and 6). Interestingly, analysis of the subcellular distribution of total viral cDNA indicates that this effect of the central DNA on total viral cDNA levels reflects primarily an accumulation of viral cDNA in the nuclear fraction; approximately 7-times more viral cDNA was found associated with the nucleus with the $cPPT^+$ virus as compared to the $cPPT^-$ virus and proportionally, 2 to 3-times more total viral cDNA was found associated to the nucleus with the wild type virus (Fig.II.6). Consistent with findings made by several previous reports using either replication-competent virus or single-round HIV-1 vector transduction systems (Follenzi, Ailles et al. 2000; Zennou, Petit et al. 2000; Maele, Rijck et al. 2003), our results indicate that the central cDNA flap enhances the establishment of HIV-1 infection by facilitating the nuclear import of proviral DNA. Moreover, our data indicate also that this process has a

stabilizing or/and a protective effect on viral cDNA. Indeed, it is possible that the central DNA flap may contribute to a correct conformation of viral cDNA and/or be implicated in the recruitment of host cell proteins to form a functional PIC capable of effective proviral DNA nuclear import. Absence of an intact central DNA flap may lead to immature PIC where viral cDNA is less stable or subject to rapid degradation. In this regard, it has recently been reported that the central DNA flap region of viral cDNA was resistant to DNAse I digestion when viral PIC complexes were isolated from the cytoplasm of infected cells at 10 hours, whereas it was sensitive to degradation when complexes were isolated at 8.5 hours after infection (Khiytani and Dimmock 2002). Altogether, these observations point toward a possible role of the central DNA flap in the maturation and/or the stability of PICs; such a role is likely to influence viral cDNA stability and nuclear import.

This positive (2-3 fold) effect of the central DNA flap on the accumulation of total viral cDNA transcripts was not observed by other studies that used lentiviral transduction systems (Follenzi, Ailles et al. 2000; Zennou, Serguera et al. 2001; Maele, Rijck et al. 2003). This difference may be due to the different target cells tested in these studies or alternatively to difference in the structure of the vector systems. For instance, the HIV-1 envelop was used for virus entry in h-PBMC in this study, while others used VSV-G pseudotyped viruses in HeLa, 293T cells and human neuroepithelial progenitors (Follenzi, Ailles et al. 2000; Zennou, Serguera et al. 2001; Maele, Rijck et al. 2003). In that regard, HIV-1 virus pseudotyped with VSV-G are known to mediate infection through distinct entry and post-entry pathways which do not entirely mimic early HIV-1 infection processes (Aiken 1997), thus possibly explaining this difference. Interestingly, a similar difference in the rate of decline of wt and cPPT-defective total viral cDNA was observed by Limon et al. (Limon, Nakajima et al. 2002) in infected PBMC although the effect was not as pronounced as in our study.

By delineating the minimal *pol* gene sequences necessary for efficient HIV-1 single-cycle replication, we have generated a RT/IN gene-deleted (2193 bp) HIV-1 vector, which can be efficiently transduced in different $CD4^+$ cells including human PBMC upon trans-complementation with Vpr-RT-IN fusion proteins. The large deletion in RT and IN gene sequence ensures that this HIV-1 vector, which expresses most HIV-1 proteins including the envelope glycoproteins, is unable to spread and replicate while at the same time minimizes possibility of generating replication-competent virus by recombination. We believe that further optimization of this HIV-1 single-cycle replication vector system and adaptation into the SIV or SHIV models will provide valuable tools to identify *in vivo* the initial target cells involved in primary virus infection as well as to evaluate precisely viral dynamics in animal models.

# Chapter III

## Contribution of the C-terminal tri-lysine regions of human immunodeficiency virus type 1 integrase for efficient reverse transcription and viral DNA nuclear import

## ABSTRACT

In addition to mediating the integration process, HIV-1 integrase (IN) has also been implicated in different steps during viral life cycle including reverse transcription and viral DNA nuclear import. Although the karyophilic property of HIV-1 IN has been well demonstrated using a variety of experimental approaches, the definition of domain(s) and/or motif(s) within the protein that mediate viral DNA nuclear import and its mechanism are still disputed and controversial. In this study, we performed mutagenic analyses to investigate the contribution of different regions in the C-terminal domain of HIV-1 IN to protein nuclear localization as well as their effects on virus infection. Our analysis showed that replacing lysine residues in two highly conserved tri-lysine regions, which are located within previously described Region C ($^{235}$WKGPAKLLWKGEGAVV) and sequence Q ($^{211}$KELQKQITK) in the C-terminal domain of HIV-1 IN, impaired protein nuclear accumulation, while mutations for $RK_{263,4}$ had no significant effect. Analysis of their effects on viral infection in a VSV-G pseudotyped RT/IN trans-complemented HIV-1 single cycle replication system revealed that all three C-terminal mutant viruses (KK215,9AA, KK240,4AE and RK263,4AA) exhibited more severe defect of induction of β-Gal positive cells and luciferase activity than an IN class I mutant D64E in HeLa-CD4-CCR5-β-Gal cells, and in dividing as well as non-dividing C8166 T cells, suggesting that some viral defects are occurring prior to viral integration. Furthermore, by analyzing viral DNA synthesis and the nucleus-associated viral DNA level, the results clearly showed that, although all three C-terminal mutants we tested inhibited viral reverse transcription to different extents, the KK240,4AE mutant exhibited most profound effect on this step, whereas KK215,9AA significantly impaired viral DNA nuclear import. In addition, our analysis could not detect viral DNA integration in each C-terminal mutant infection, even though they displayed

various low levels of nucleus-associated viral DNA, suggesting that these C-terminal mutants also impaired viral DNA integration ability. All of these results indicate that, in addition to being involved in HIV-1 reverse transcription and integration, the C-terminal tri-lysine regions of IN also contribute to efficient viral DNA nuclear import during the early stage of HIV-1 replication.

## INTRODUCTION

The integrase (IN) of human immunodeficiency virus type 1 (HIV-1) is encoded by the *pol* gene and catalyzes integration of viral cDNA into host chromosome, an essential step in HIV-1 replication. In addition to mediating the integration process, HIV-1 IN also participates in different steps during viral life cycle, including reverse transcription and viral DNA nuclear import (Engelman and Craigie 1995; Engelman, Englund et al. 1995; Bukovsky and Gottlinger 1996; Gallay, Hope et al. 1997; Nakamura, Masuda et al. 1997; Wu, Liu et al. 1999; Ikeda, Nishitsuji et al. 2004). During early phase of the HIV-1 replication cycle, after virus entry into target cells, another *pol* gene product, reverse transcriptase (RT), copies viral genomic RNA into double-stranded cDNA which exists within a nucleoprotein preintegration complex (PIC). The PIC also contains viral proteins including RT, IN, nucleocapsid (NC, p9), Vpr and matrix (MA, p17) and this large nucleoprotein complex is capable of actively translocating into the cell nucleus, including that of non-dividing cells (reviewed in reference (Piller, Caly et al. 2003). This feature is particularly important for the establishment of HIV-1 replication and pathogenesis in exposed hosts, since the infection of postmitotic cells including tissue macrophages, mucosal dendritic cells as well as non-dividing T cells may be essential not only for viral transmission and dissemination, but also for the establishment of persistent viral reservoirs.

HIV-1 IN is composed of three functional domains, an N-terminal domain, a central catalytic core domain and a C-terminal domain, all of which are required

for a complete integration reaction. The N-terminal domain harbors an HHCC-type zinc binding domain and is implicated in the multimerization of the protein and contributes to the specific recognition of DNA ends (Ellison, Gerton et al. 1995; Lee, Xiao et al. 1997; Heuer and Brown 1998). The core domain of IN contains the highly conserved DDE motif which is important for catalytic activity of the protein (Engelman and Craigie 1992; van Gent, Groeneger et al. 1992). The C-terminal domain was shown to possess nonspecific DNA binding properties (Eijkelenboom, Lutzke et al. 1995; Lutzke and Plasterk 1998). Some mutations within this region cause a drastic loss of virus infectivity without affecting the enzymatic activity of IN *in vitro* (Eijkelenboom, Lutzke et al. 1995; Engelman, Englund et al. 1995; Wiskerchen and Muesing 1995; Lutzke and Plasterk 1998; Lu, Limon et al. 2004). There are three conserved sequences in the C-terminus of IN that are essential for HIV-1 replication. Regions C ($^{235}$WKGPAKLLWKGEGAVV) and N ($^{259}$VVPRRKAK) are conserved in all known retroviruses and the $^{211}$KELQKQITK motif falls within the so–called glutamine-rich based region (sequence Q) of lentiviruses (Cannon, Byles et al. 1996). Alteration of each of the three sequences such as Q214L/Q216L, K215A/K219A, W235E, K236A/K240A, K244A/E246A, RRE263-5AAH resulted in loss of viral replication (Wiskerchen and Muesing 1995; Cannon, Byles et al. 1996; Petit, Schwartz et al. 2000; Lu, Limon et al. 2004). However, the mechanism(s) underlying the loss of viral infectivity remains controversial.

A number of studies have demonstrated the karyophilic properties of IN implicating that this protein may play an important role for PIC nuclear import (Gallay, Hope et al. 1997; Petit, Schwartz et al. 1999; Pluymers, Cherepanov et al. 1999; Tsurutani, Kubo et al. 2000; Bouyac-Bertoia, Dvorin et al. 2001; Depienne, Mousnier et al. 2001). However, the definition of nuclear localization signals (NLSs) in IN as well as their contribution to HIV-1 PIC nuclear import still remains to be determined. Previous report has suggested an atypical bipartite NLS ($^{186}$KRK and $^{211}$KELQKQITK) by showing that IN mutants K186Q and

Q214/216L in these regions lost the protein nuclear localization and their ability to bind to karyopherin α *in vitro* (Gallay, Hope et al. 1997). However, in attempt to analyze the effect of these mutants during HIV-1 replication, other studies did not reveal the importance of these IN mutants (K186Q and Q214/216L) for viral nuclear import; rather they appear to be required for reverse transcription, integration or undefined post-nuclear entry steps (Petit, Schwartz et al. 2000; Tsurutani, Kubo et al. 2000; Lu, Limon et al. 2004). Also, another IN amino acid sequence IIGQVRDQAEHLK (aa161-173), was initially identified as an atypical NLS, which is required for viral DNA nuclear import (Bouyac-Bertoia, Dvorin et al. 2001). However, reassessments of this putative NLS function failed to confirm this conclusion (Dvorin, Bell et al. 2002; Limon, Devroe et al. 2002). Some reports have also acknowledged that IN localization could result from passive diffusion of the protein and its DNA binding property (Kukolj, Jones et al. 1997; Devroe, Engelman et al. 2003), but DNA binding alone does not fully explain a rapid, ATP- and temperature-dependent nuclear import of IN (Depienne, Mousnier et al. 2001). It has recently been reported that the nuclear translocation of HIV-1 IN can be attributed to its interaction with a cellular component, human lens epithelium-derived growth factor/transcription coactivator p75 (LEDGF/p75) and LEDGF/p75 was also shown to be a component of HIV PIC (Maertens, Cherepanov et al. 2003; Llano, Vanegas et al. 2004). However, whether this IN/LEDGF/p75 interaction plays an important role for HIV-1 nuclear import still remains to be elucidated, since HIV-1 infection and replication in LEDGF/p75-deficient cells was equivalent to that in control cells, regardless whether cells were dividing or growth arrested (Llano, Vanegas et al. 2004). Thus, even though extensive studies have been dedicated in this specific research field, the contribution of HIV-1 IN to viral PIC nuclear import remains to be defined.

In this study, we have performed substitution mutational analysis to investigate the contribution of different C-terminal regions of IN to protein nuclear localization and their effects on HIV-1 replication. Our results showed

that mutations of lysine residues in two tri-lysine regions, which are located within previously described Region C and sequence Q (Cannon, Byles et al. 1996) in the C-terminal domain of HIV-1 IN, impaired protein nuclear localization, while mutations of arginines at amino acid position of 263 and 264 in the distal part of the C-terminal domain of IN had no significant effect. Moreover, we assessed the effect of these IN mutants during HIV-1 single cycle infection mediated by VSV-G pseudotyped RT/IN trans-complemented viruses. Results showed that, while all three C-terminal mutant viruses differentially affected HIV-1 reverse transcription, the KK240,4AE mutant exhibited most profound inhibition on this step, whereas KK215,9AA significantly impaired viral DNA nuclear import.

## MATERIAL AND METHODS

**Construction of different IN expressors and HIV-1 RT/IN defective provirus.** The full-length wild-type HIV-1 IN cDNA was amplified by polymerase chain reaction (PCR) using HIV-1 HxBru strain (Yao, Subbramanian et al. 1995) as template and an engineered initiation codon (ATG) was placed prior to the first amino acid (aa) of IN. The primers are 5'-IN-HindIII-ATG (5'-GCGCAAGCTTGGATAGATGTTTTTAGATGGAA-3') and 3'-IN-Asp718 (5'-CCATGTGTGGTACCTCATCCTGCT-3'). The PCR product was digested with *Hind*III and *Asp*718 restriction enzymes and cloned in frame to 5' end of EYFP cDNA in a pEYFP-N1 vector (BD Biosciences Clontech) and generated a IN-YFP fusion expressor. Also, cDNA encoding for truncated IN (aa 50 to 288 or aa 1 to 212) was amplified by PCR and also cloned into pEYFP-N1 vector. The primers for generation of IN50-288 cDNA are IN50-HindIII-ATG-5'(5'-GCGCAAGCTTGGATAGATGCATGGACAAGTAG-3) and 3'-IN-Asp718 and primers for amplifying IN1-212 cDNA are IN-HindIII-ATG-5' and IN-212-XmaI-3'(5'-CAATTCCCGGGTTTGTATGTCTGTTTGC-3). IN substitution mutants $IN_{KK215,9AA}$-YFP, $IN_{KK240,4AE}$-YFP and $IN_{RK263,4AA}$-YFP, were generated

by a two-step PCR-based method (Yao, Kobinger et al. 1999) by using a 5'-primer (5'-IN-HindIII-ATG), a 3'-primer (3'-IN-Asp718) and complementary primers containing desired mutations. Amplified IN cDNAs harboring specific mutations were then cloned into pEYFP-N1 vector. To improve the expression of each IN-YFP fusion protein, all IN-YFP fusing cDNAs were finally subcloned into a SVCMV vector, which contains a cytomegalovirus (CMV) immediate early gene promoter (Yao, Kobinger et al. 1999).

To construct HIV-1 RT/IN defective provirus NLlucΔBglΔRI, we used a previously described HIV-1 envelope-deleted NLlucΔBglD64E provirus as the backbone (kindly provided by Dr. Irvin S.Y. Chen). In this provirus, the *nef* gene was replaced by a firefly luciferase gene (Poon and Chen 2003). The *ApaI*/*SalI* cDNA fragment in NLlucBglD64E was replaced by the corresponding fragment derived from a HIV-1 RT/IN deleted provirus $R^-$/ΔRI (Ao, Yao et al. 2004) and generated a RT/IN deleted provirus NLlucΔBglΔRI, in which RT and IN gene sequences were deleted while a 194-bp sequence harboring cPPT/CTS *cis*-acting elements was maintained. To restore HIV-1 envelope gene sequence in NLlucΔBglΔRI provirus, the *SalI*/*BamH*I cDNA fragment in this provirus was replaced by a corresponding cDNA fragment from a HIV-1 envelope competent provirus $R^-$/ΔRI (Ao, Yao et al. 2004) and the resulting provirus is named as NLlucΔRI. To functionally complement RT/IN defects of NLlucΔBglΔRI, a CMV-Vpr-RT-IN fusion protein expressor (Ao, Yao et al. 2004) was used in this study. Co-transfection of NLlucΔBglΔRI, CMV-Vpr-RT-IN and a vesicular stomatitis virus G (VSV-G) glycoprotein expressor results in the production of VSV-G pseudotyped HIV-1 that can undergo for single cycle replication in different cell types (Ao, Yao et al. 2004). To investigate the effect of IN mutants on viral replication, different mutants KK215,9AA, KK240.4AE, RK263,4AA or D64E were introduced into CMV-Vpr-RT-IN expressor by PCR-based method as described above and using a 5'-primer corresponding to a sequence in RT gene

and including a natural *Nhe*I site (5'-GCAGCTAGCAGGGAGACTAA-3'), a 3'-primer (3'-IN-stop-*Pst*I, 5'- CTGTTCCTGCAGCTAATCCTCATCCTG-3') and the complementary oligonucleotide primers containing desired mutations. All IN mutants were subsequently analyzed by DNA sequencing to confirm the presence of mutations or deletions.

**Cell lines and reagents.** Human embryonic kidney 293T, HeLa and HeLa-CD4-CCR5-β-Gal cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Human C8166 T-lymphoid cells were maintained in RPMI-1640 medium. Antibodies used in the immunofluorescent assay, immunoprecipitation or western blot are as follows: The HIV-1 positive human serum 162 and anti-HIVp24 monoclonal antibody used in this study were previously described (Yao, Mouland et al. 1998). The rabbit anti-GFP and anti-IN antibodies were respectively obtained from Molecular Probes Inc and through AIDS Research Reference Reagent Program, Division of AIDS, NIAID, NIH. Aphidicolin was obtained from Sigma Inc.

**Cell transfection and immunofluorescence assay.** DNA transfection in 293T and HeLa cells were performed with standard calcium phosphate DNA precipitation method. For immunofluorescence analysis, HeLa cells were grown on glass coverslip (12 $mm^2$) in 24-well plate. After 48h of transfection, cells on the coverslip were fixed with PBS-4% paraformaldehyde for 5 minutes, permeabilized in PBS-0.2% Triton X-100 for 5 minutes and incubated with primary antibodies specific for GFP or HIV-1 IN followed by corresponding secondary FITC-conjugated antibodies. Then, cells on the coverslip were viewed using a computerized Axiovert 200 inverted fluorescence microscopy (Becton Deckson Inc).

**Virus production and infection.** Production of different single-cycle replicating virus stocks and measurement of virus titer were previously described (Ao, Yao et al. 2004). Briefly, 293T cells were co-transfected with RT/IN defective NLlucΔBglΔRI provius, a VSV-G expressor and each of CMV-Vpr-RT-

IN (wt/mutant) expressor. To produce HIV-1 envelope competent single cycle replicating virus, 293T cells were co-transfected with NLlucΔRI and different CMV-Vpr-RT-IN (wt/mutant) expressors. After 48 hours of transfection, supernatants were collected and virus titers were quantified by RT activity assay (Yao, Kobinger et al. 1999).

To test the effect of IN mutants on virus infection, equal amounts of virus were used to infect HeLa-CCR5-CD4-β-Gal cells, dividing and non-dividing C8166 T cells. To compare the infection of each viral stock in HeLa-CCR5-CD4-β-Gal cells, numbers of infected cells (β-Gal positive cells) were evaluated by the MAGI assay 48 hours post-infection (p.i.) as described previously (Kimpton and Emerman 1992). To infect CD4+ T cells, dividing or aphidicolin-treated non-dividing C8166 T cells (with 1.3 μg/ml of aphidicolin) were infected with equivalent amounts of single cycle replicating viruses (5cpm/cell) for 2 hours. Then, infected cells were washed and cultured in the absence or presence of the same concentration of aphidicolin. At 48 hours post-infection, $1x10^6$ cells from each sample were collected, washed twice with PBS, lysed with 50 μl of luciferase lysis buffer (Fisher Scientific Inc) and then, 10 μl of cell lysate was subjected to the luciferase assay by using a TopCount®NXT™ Microplate Scintillation &Luminescence Counter (Packard, Meriden) and the luciferase activity was valued as relative luciferase units (RLU). Each sample was analyzed in duplicate and the average deviation was calculated.

**Immunoprecipitation and Western blot analyses.** For detection of IN-YFP fusion proteins, 293T cells transfected with each IN-YFP expressor were lysed with RIPA lysis buffer and immunoprecipitated using human anti-HIV serum. Then, immunoprecipitates were run in 12% SDS-PAGE and analyzed by Western blot using rabbit anti-GFP antibody. To analyze virion-incorporation of IN and virus composition, 293T cells were co-transfected with NLlucΔBglΔRI provirus and each of CMV-Vpr-RT-IN (wt/mutant) expressors. After 48 hours,

viruses were collected, lysed with RIPA lysis buffer and immunoprecipitated with human anti-HIV serum. Then, immunoprecipitates were run in 12% SDS-PAGE and analyzed by Western blot with rabbit anti-IN antibody and anti-p24 monoclonal antibody.

**HIV-1 reverse-transcribed and integrated DNA detection by PCR and Southern blotting.** C8166 T cells were infected with equal amount of the wt or IN mutant viruses for 2 hours, washed for three times and cultured in RPMI medium. To detect total viral DNA synthesis, at 12 hours post-infection, equal number ($1x10^6$ cells) of cells were collected, washed twice with PCR washing buffer (20mM Tris-HCl, pH8.0, 100mM KCl), and lysed in lysis buffer (PCR washing buffer containing 0.05% NP-40, 0.05% Tween-20). Lysates were then incubated at 56°C for 30 min with proteinase K (100 μg/ml) and at 90°C for 10 min prior to phenol-chloroform DNA purification. To detect viral cDNA from each sample, all lysates were serially diluted 5-fold and subjected to PCR analysis. The primers used to detect late reverse transcription products were as following: 5'-LTR-U3, 5'-GGATGGTGCTTCAAGCTAGTACC-3' (nt position 8807, +1= start of BRU of transcription initiation); 3'-Gag 5'-ACTGACGCTCTCGCACCCATCTCTCTC-3' (nt position 329). The probe for southern blot detection was generated by PCR with a 5'-LTR-U5 oligonucleotide, 5'-CTCTAGCAGTGGCGCCCGAACAGGGAC-3' (nt position 173) and the 3'-Gag oligo. PCR was carried out using 1x HotStar Taq Master Mix kit (QIAGEN, Mississauga, Ontario), as described previously (Ao, Yao et al. 2004).

To analyze nucleus- and cytoplasm-associated viral DNA, a subcellular fractionation of infected C8166 T cells ($2x10^6$) was performed after 24 hours of infection, as described previously (Simon and Malim 1996). Briefly, infected cells were pelleted and resuspended in ice-cold PCR lysis buffer (washing buffer containing 0,01% NP-40). After a 5-min incubation on ice, the nucleus was pelleted by centrifugation, washed twice with PCR wash buffer, and lysed in lysis buffer (0,05% NP-40, 0,05% Tween-20). Then, both cytoplasmic sample

(supernatant from the first centrifugation) and the nuclear sample were treated with proteinase K and used for PCR analysis, as described above.

Integrated proviral DNA was detected in cell lysates by a modified nested Alu-PCR (Ao, Yao et al. 2004), in which following the first PCR, a second PCR was carried-out to amplify a portion of the HIV-1 LTR sequence from the first Alu-LTR PCR-amplified products. The first PCR was carried out by using primers including 5'-Alu oligo (5'-TCCCAGCTACTCGGGAGGCTGAGG-3') and 3'-LTR oligo (5'-AGGCAAGCTTTATTGAGGGCTTAAGC-3') (nt position 9194) located respectively in the conserved region of human Alu sequence and in HIV-1 LTR. The primer used for both of the second nested PCR and for generating a probe are 5'-NI: 5'-CACACACAAGGCTACTTCCCT-3' and 3'-NI: 5'-GCCACTCCCCAGTCCCGCCC-3'. As a control, the first and second PCR primer pairs were also used in parallel to detect integrated viral DNA from serially diluted ACH-2 cells, which contain one viral copy/cell, in a background of uninfected C8166 cellular DNA.

To evaluate the DNA content of extracted chromosomal DNA preparations, detection of human β-globin gene was carried-out by PCR, as described previously (Simon and Malim 1996). All final PCR products were electrophoresed through 1.2% agarose gel and transferred to hybridization transfer membrane (GeneScreen Plus, PerkinElmer Life Sciences), subjected to Southern hybridization by using specific PCR DIG-Labeling probes (Roche Diagnostics, Laval, Que) and visualized by a chemiluminescent method. Densitometric analysis was performed using a Personal Molecular Imager (Bio-Rad) and Quantity One software version 4.1.

## RESULTS

### 1. The C-terminal domain of HIV-1 integrase (IN) is required for the nuclear localization of IN-YFP fusion protein.

In this study, we first investigated the intracellular localization of HIV-1 IN and delineated the region(s) of IN contributing to its karyophilic property. A HIV-1 IN-YFP fusion protein expressor (CMV-IN-YFP) was generated by fusing a full-length HIV-1 IN cDNA (amplified from HIV-1 HxBru molecular clone (Yao, Subbramanian et al. 1995) to the 5' end of YFP cDNA in a CMV-IN-YFP expressor, as described in Materials and Methods. Transfection of CMV-IN-YFP expressor in 293T cells resulted in the expression of a 57 kDa IN-YFP fusion protein (Fig.III.1B, lane 2; Fig. 2B, lane 1), whereas expression of YFP alone resulted in a 27 kDa protein (Fig.III.2B, lane 5). Given that HeLa cells have well-defined morphology and are suitable for observation of intracellular protein distribution, we tested the intracellular localization of YFP and IN-YFP by transfecting CMV-IN-YFP or CMV-YFP expressor in HeLa cells. After 48 hours of transfection, cells were fixed and subjected to indirect immunofluorescence assay using primary rabbit anti-GFP antibody followed by secondary FITC-conjugated anti-rabbit antibodies. Results showed that, in contrast to a diffused intracellular localization pattern of YFP (data not shown), the IN-YFP fusion protein was predominantly localized in the nucleus (Fig.III.1C, a1), confirming the karyophilic feature of HIV-1 IN.

To delineate the karyophilic determinant in HIV-1 IN, two truncated IN-YFP expressors CMV-$IN_{50-288}$-YFP and CMV-$IN_{1-212}$-YFP were generated. In CMV-$IN_{50-288}$-YFP, the N-terminal HH-CC domain of IN (aa 1-49) was deleted and in CMV-$IN_{1-212}$-YFP, the C-terminal domain (aa 213-288) was removed (Fig.III.1A). Transfection of each truncated IN-YFP fusion protein expressor in 293T cells resulted in the expression of $IN_{50-288}$-YFP and $IN_{1-212}$-YFP at approximately 52 kDa and 48 kDa molecular mass respectively (Fig.III.1B, lanes 3 and 4). We next investigated the intracellular localization of truncated IN-YFP fusion proteins in HeLa cells by using indirect immunofluorescence assay, as described above. Results showed that the $IN_{50-288}$-YFP was predominantly localized in the nucleus with a similar pattern as the wild-type IN- YFP fusion

protein (Fig.III.1C, compare b1 to a1). However, $IN_{1-212}$-YFP fusion protein was excluded from the nucleus, with an accumulation of the mutant protein in the cytoplasm (Fig.III. 1C, c1). These results were also further confirmed by using rabbit anti-IN antibody immunofluorescence assay (data not shown). Taken together, our data show that the C-terminal domain of HIV-1 IN is required for its nuclear accumulation.

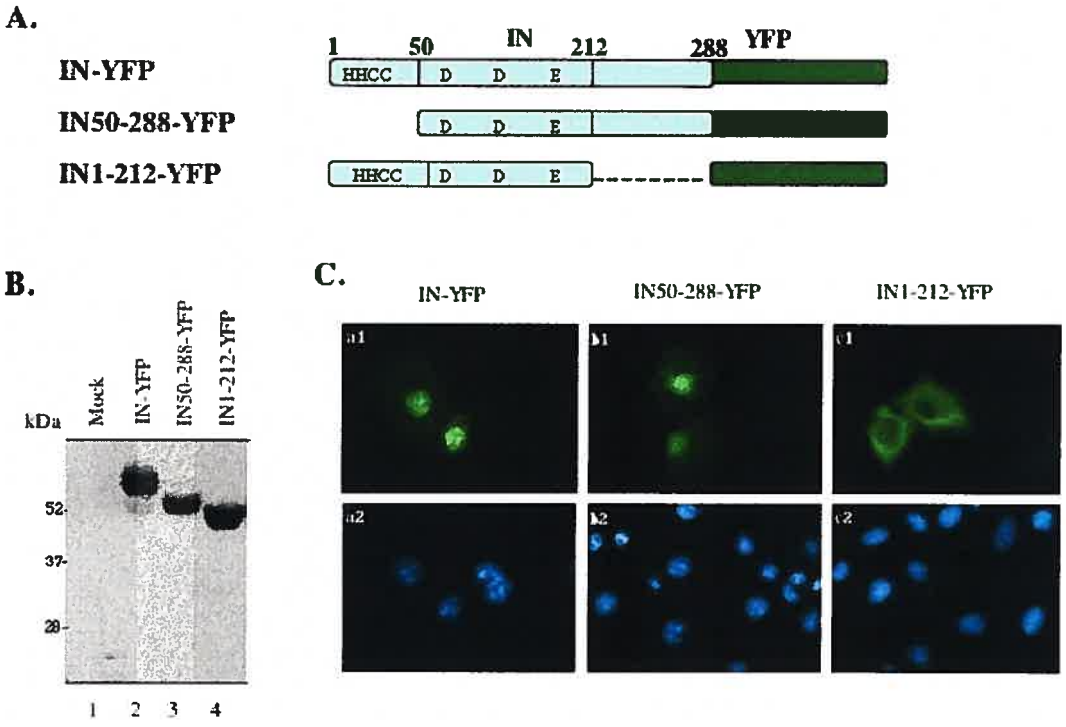


**Fig.III.1. Subcellular localization of the wild-type and truncated HIV integrase fused with YFP. A) Schematic structure of HIV-1 integrase–YFP fusion proteins.** Full-length (1-288aa) HIV-1 integrase, the N-terminus-truncated mutant (51-228aa) or the C-terminus-truncated mutant (1-212aa) was fused in frame at the N-terminus of YFP protein. The cDNA encoding for each IN-YFP fusion protein was inserted in a SVCMV expression plasmid. **B) Expression of different IN-YFP fusion proteins in 293T cells**. 293T cells were transfected with each IN-YFP expressor and at 48 hours of transfection, cells were lysed, immunoprecipitated with anti-HIV serum and resolved by electrophoresis through a 12.5% SDS-PAGE followed by Western blot with rabbit anti-GFP antibody. The molecular weight markers are indicated at the left side of the gel. **C) Intracellular localization of different IN-YFP fusion proteins.** HeLa cells

were transfected with each HIV-1 IN-YFP fusion protein expressor and at 48 hours of transfection, cells were fixed and subjected to indirect immunofluorescence using rabbit anti-GFP and then incubated with FITC-conjugated anti-rabbit antibodies. The localization of each fusion protein was viewed by Fluorescence microscopy with a 50X oil immersion objective. Upper panel is fluorescence images and bottom panel is DAPI nucleus staining.

**2. Two tri-lysine regions in the C-terminal domain of IN are involved in the protein nuclear localization.**

The C-terminal domain of HIV-1 IN contains several regions that are highly conserved in different HIV-1 strains, including Q, C and N regions (Cannon, Byles et al. 1996). Interestingly, in regions Q and C, sequences of $^{211}$KELQKQITK and $^{236}$KGPAKLLWK possess high similarity in terms of numbers and position of lysine residues and therefore, we term them proximal tri-lysine region and distal tri-lysine region, respectively (Fig.III.2A). All of these lysine residues are highly conserved in most HIV-1 strains (Kuiken, Foly et al. 2001). To test whether these basic lysine residues could constitute for a possible nuclear localization signal for IN nuclear localization, we specifically introduced substitution mutations for two lysines in each tri-lysine region and generated $IN_{KK215,9AA}$-YFP and $IN_{KK240,4AE}$-YFP expressors (Fig.III.2A). In the conserved N region, there is a stretch of four basic residues among five amino acids (aa) $^{262}$RRKAK. To characterize whether this basic aa region may contributes to IN nuclear localization, we replaced an arginine and a lysine at positions of 263 and 264 by alanines in this region and generated a mutant ($IN_{RK263,4AA}$-YFP). The protein expression of different IN-YFP mutants in 293T cells showed that, like the wild type IN-YFP, each IN-YFP mutant fusion protein was detected at similar molecular mass (57 kDa) in SDS-PAGE (Fig.III.2B, lanes 1 to 4), while YFP

alone was detected at position of 27 kDa (lane 5). Then, the intracellular

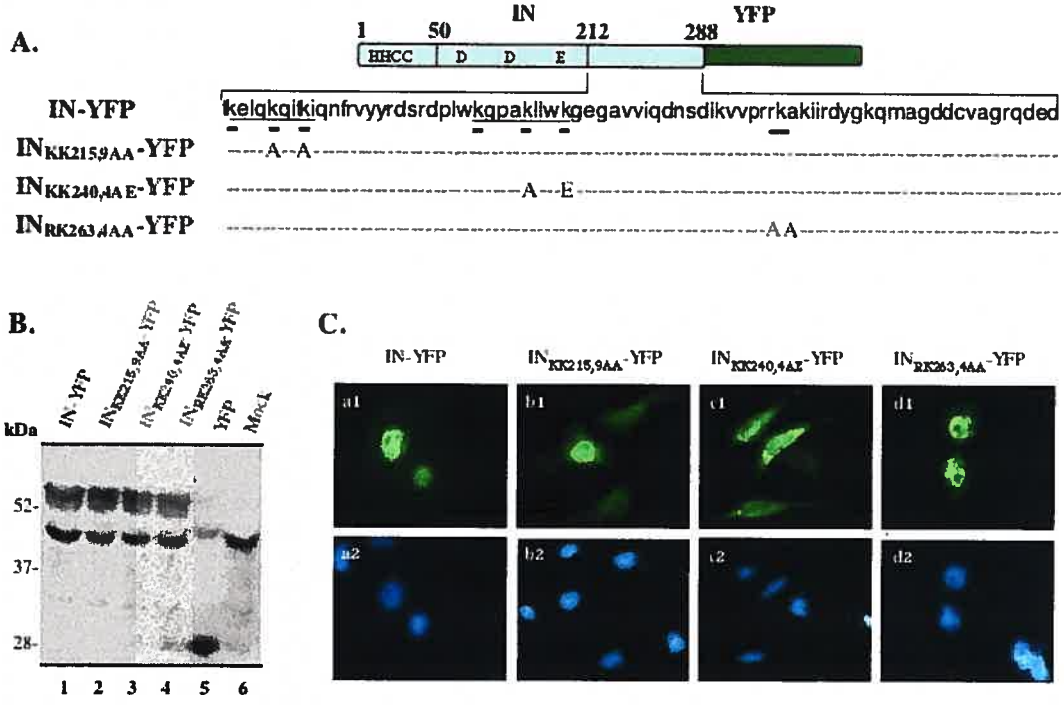


**Fig.III.2. Effect of different IN C-terminal substitution mutants on IN-YFP intracellular localization.** **A)** Diagram of HIV-1 IN domain structure and introduced mutations at the C-terminal domain of the protein. The position of lysines in two tri-lysine regions and introduced mutations are shown at the bottom of sequence. **B)** The expression of the wild-type and mutant IN-YFP fusion proteins were detected in transfected 293T cells by using immunoprecipitation with anti-HIV serum and Western blot with rabbit anti-GFP antibody, as described in figure 1. The molecular weight markers are indicated at the left side of the gel. **C)** Intracellular localization of different HIV-1 IN mutant-YFP fusion proteins in HeLa cells were analyzed by fluorescence microscopy with a 50X oil immersion objective. The nucleus of HeLa cells was simultaneously visualized by DAPI staining (lower panel).

localization of each IN mutant was investigated in HeLa cells by using similar methods, as described above. Results showed that, while the wild type IN-YFP and $IN_{RK263,4AA}$-YFP still predominantly localized to the nucleus (Fig.III.2C, a1 and d1), both $IN_{KK215,9AA}$-YFP and $IN_{KK240,4AE}$-YFP fusion proteins were shown to distribute throughout the cytoplasm and nucleus, but with much less intensity

in the nucleus (Fig.III.2C, a1 and b1). These data suggest that these lysine residues in each tri-lysine regions are required for efficient HIV-1 IN nuclear localization.

**3. Production of VSV-G pseudotyped HIV-1 IN mutant viruses and their effects on HIV-1 infection.**

Given that two di-lysine mutants located in the C-terminal domain of IN are involved in HIV-1 IN nuclear localization, we next evaluated whether these IN mutants would affect the efficiency of HIV-1 infection. To specifically analyze the effect of IN mutants in early steps of viral infection, we modified a previously described HIV-1 single-cycle replication system (Ao, Yao et al. 2004) and constructed a RT/IN/Env gene-deleted HIV-1 provirus NLlucΔBglΔRI, in which the *nef* gene was replaced by a firefly luciferase gene (Poon and Chen 2003). Co-expression of NLlucΔBglΔRI provirus with Vpr-RT-IN expressor and a vesicular stomatitis virus G (VSV-G) glycoprotein expressor will produce viral particles that can undergo a single-round of replication, since RT, IN and Env defects of provirus will be complemented *in trans* by VSV-G glycoprotein and Vpr-mediated RT and IN trans-incorporation (Ao, Yao et al. 2004). This single cycle replication system allows us to introduce different mutations into IN gene sequence without differentially affecting viral morphogenesis and the activity of the central DNA Flap. After different IN mutations KK215,9AA, KK240,4AE and RR263,4AA were introduced into Vpr-RT-IN expressor, we produced VSV-G pseudotyped HIV-1 IN mutant virus stocks in 293T cells. In order to specifically investigate the effect of IN mutants on early steps during HIV-1 infection prior to integration, an IN class I mutant D64E was also included as control. After each viral stock was produced (as indicated in Fig.III.3A), similar amounts of each virus stock (quantified by virion-associated RT activity) were lysed and virus composition and trans-incorporation of RT and IN of each virus stock were analyzed by Western blot analysis with anti-IN and anti-HIV

antibodies, as described in Materials and Methods. Results showed that all VSV-G pseudotyped IN mutant viruses had similar levels of Gagp24, IN and RT, as compared to the wild-type virus (Fig.III.3A), indicating that trans-incorporation of RT and IN as well as HIV-1 Gag processing were not differentially affected by the introduced IN mutations.

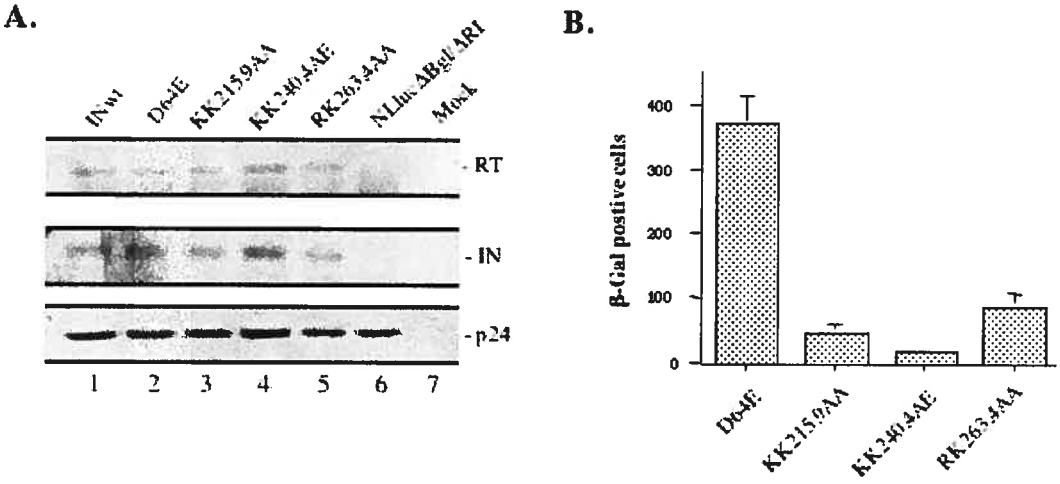


**Fig.III.3. Production of different single-cycle replicating viruses and their infection in HeLa-CD4-CCR5-β-Gal cells. A).** To evaluate the trans-incorporation of RT and IN in VSV-G pseudotyped viral particles, viruses released from 293T cells transfected with NLlucΔBglΔRI provirus alone (lane 6) or cotransfected with different Vpr-RT-IN expressors and a VSV-G expressor (lane 1 to 5) were lysed, immunoprecipitated with anti-HIV serum. Then, immunoprecipitates were run in 12% SDS-PAGE and analyzed by Western blot with rabbit anti-IN antibody (middle panel) or anti-RT and anti-p24 monoclonal antibody (upper and lower panel). **B).** The infectivity of trans-complemented viruses produced in 293 T cells was evaluated by MAGI assay. HeLa-CD4-CCR5-LTR-β-Gal cells were infected with equal amounts (at 10 cpm/cell) of different IN mutant viruses and after 48 hours of infection, numbers of β-Gal positive cells (infected cell) were monitored by X-gal staining. Error bars represent variation between duplicate samples and the data is representative of results obtained in three independent experiments.

To test the infectivity of different IN mutant viruses in HeLa-CD4-CCR5-LTR-β-Gal cells, we first compared the infectivity of VSV-G pseudotyped wild type virus and the D64E mutant virus. At 48 hours post-infection with equivalent amount of each virus stock (at 1 cpm RT activity/cell), the number of β-Gal

positive cells was evaluated by MAGI assay, as described previously (Kimpton and Emerman 1992). Results showed that the number of infected cells (β-Gal positive cells) for D64E mutant reached approximately 14% of the wild type level (data not shown). This result is consistent with a previous report showing that, in HeLa MAGI assay, the infectivity level of class I IN integration-defect mutant was approximately 20 to 22% of wild type level (Wiskerchen and Muesing 1995). It indicates that, even though the IN mutant D64E virus is defective for integrating viral DNA into host genome, *tat* expression from nucleus-associated and unintegrated viral DNAs can activate HIV-1 LTR-driven β-Gal expression in HeLa-CD4-CCR5-LTR-β-Gal cells. Indeed, several studies have already shown that HIV infection leads to selective transcription of *tat* and *nef* genes before integration (Engelman, Englund et al. 1995; Wu and Marsh 2001; Brussel and Sonigo 2004). Therefore, this HeLa-CD4-CCR5-LTR-β-Gal cell infection system provides an ideal method for us to evaluate the effect of different IN mutants on early steps of viral infection prior to integration. We next infected HeLa-CD4-CCR5-LTR-β-Gal cells with different VSV-G pseudotyped IN mutant viruses at higher infection dose of 10 cpm RT activity/cell and numbers of β-Gal positive cells were evaluated by MAGI assay after 48 hours of infection. Interestingly, results showed that the IN mutant D64E virus infection induced the highest level of β-Gal positive cells, whereas infection with viruses containing IN mutants KK215,9AA, KK240,4AE or RK263,4AA yielded much lower levels of β-Gal positive cells, which only reached approximately 11%, 5% or 26% of the level of D64E virus infection (Fig.III.3B). Based on these results, we reasoned that these IN C-terminal mutants blocked infection mostly by affecting earlier steps of HIV-1 life cycle, such as reverse transcription and/or viral DNA nuclear import steps, which are different from the action of D64E mutant on viral DNA integration.

**4. Effect of IN mutants on viral infection in dividing and non-dividing C8166 T cells.**

To further test whether these C-terminal mutants could induce similar phenotypes in $CD4^+$ T cells, we infected dividing and non-dividing (aphidicolin-treated) C8166 $CD4^+$ T cells with equal amounts of VSV-G pseudotyped IN mutant viruses (at 5 cpm of RT activity/cell). Since all IN mutant viruses contain a luciferase (luc) gene in place of the *nef* gene, viral infection can be monitored by using a sensitive luc assay which could efficiently detect viral gene expression from integrated and unintegrated viral DNA (Poon and Chen 2003). After 48 hours of infection, equal amounts of cells were lysed in 50 μl of luc lysis buffer and then, 10 μl of cell lysates was used for measurement of luc activity, as described in Materials and Methods. Results showed that the D64E mutant infection in dividing C8166 T cells induced $14.3x10^4$ RLU of luc activity (Fig.III.4 A), which was approximately 1000-fold lower than that in the wild type virus infection (data not shown). This level of luc activity detected in D64E mutant infection is mostly due to *nef* gene expression from the unintegrated DNA (Poon and Chen 2003). In agreement with the finding by MAGI assay described in figure 3, the Luc activity detected in KK215,9AA, KK240,4AE and RK263,4AA mutant samples were approximately 13%, 5% and 36% of level of D64E mutant infection (Fig.III.4A). In parallel, infection of different IN mutants in non-dividing C8166 T cells was also evaluated and similar results were observed (Fig.III.4B).

To test whether these IN mutants had similar effects during HIV-1 envelope-mediated single cycle infection, we produced virus stocks by co-transfecting 293T cells with a HIV-1 envelope-competent NLlucΔRI provirus with each Vpr-RT-IN mutant expressor, as described in Materials and Methods. Then, dividing $CD4^+$ C8166 cells were infected with each virus stock (at 10 cpm RT activity/cells). At 48 hours post-infection, cells were collected and measured for luc activity. Results from figure III.4C showed that, similar to results obtained from VSV-G pseudotyped virus infection (Fig.III.4A), the Luc activity detected in cells infected by HIV-1 envelope competent KK215,9AA, KK240,4AE and

RK263,4AA mutant viruses were approximately 13.5%, 6% and 29% of level of D64E mutant infection (Fig.III.4C). All of these results confirm the data from HeLa-CD4-CCR5-LTR-β-Gal infection (Fig.III.3) by using either VSV-G- and HIV-1 envelope-mediated infections and suggest again that the significantly attenuated infection of KK215,9AA, KK240,4AE and RK263,4AA mutant viruses may be due to their defect(s) at reverse transcription and/or viral DNA nuclear import steps.

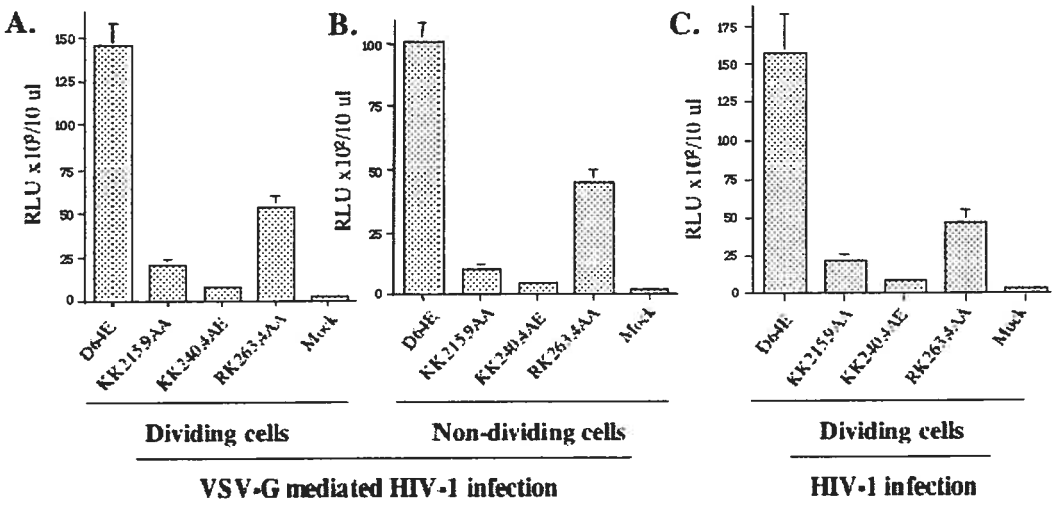


**Fig.III.4. Effect of IN mutants on viral infection in dividing and nondividing C8166 T cells.** To test the effect of different IN mutants on HIV-1 infection in CD4+ T cells, dividing (panel A) and non-dividing (aphidicolin-treated, panel B) C8166 T cells were infected with equal amount of VSV-G pseudotyped IN mutant viruses (at 5 cpm/cell). For evaluation of the effect of different IN mutants on HIV-1 envelope-mediated infection in CD4+ T cells, dividing C8166 T cells were infected with equal amount of HIV-1 envelope competent IN mutant viruses (at 10 cpm/cell) (panel C). After 48 hours of infection, HIV-1 DNA-mediated luciferase induction was monitored by luciferase assay. Briefly, the same amount ($10^6$ cells) of cells was lysed in 50 ul of luciferase lysis buffer and then, 10 μl of cell lysate was subjected to the luciferase assay. Error bars represent variation between duplicate samples and the data is representative of results obtained in three independent experiments.

**5. Effects of IN mutants on reverse transcription, viral DNA nuclear import and integration.**

All results so far suggest that these C-terminal mutants might significantly affect early steps during HIV-1 replication. To directly assess the effect of these IN C-terminal mutants on each early step during viral infection, we analyzed the viral DNA synthesis, their nuclear translocation and integration following each IN mutant infection in dividing C8166 cells. Levels of HIV-1 late reverse transcription products were analyzed by semi-quantitative PCR after 12 hours of infection with HIV-1 specific 5'-LTR-U3/3'-Gag primers and Southern blot, as previously described (Simon and Malim 1996; Ao, Yao et al. 2004). Also, intensity of amplified HIV-1 specific DNA in each sample was evaluated by laser densitometric scanning of bands in Southern blot autoradiograms (Fig.III.5A). Results showed that total viral DNA synthesis in both KK215,9AA and RK263,4AA infection reached approximately 61% and 46% of that of the wild type (wt) virus infection (Fig.III.5A and B). Strikingly, in KK240,4AA sample, detection of viral DNA synthesis was drastically reduced, which only reached 21% of viral DNA level in WT sample (Fig.III.5A and B). These results indicate that all three C-terminal mutants negatively affected viral reverse transcription during viral infection and KK240,4AA mutant exhibited most profound effect.

Meanwhile, the nucleus- and cytoplasm-associated viral DNA levels were analyzed at 24 hours post-infection in C8166 T cells. The infected cells were first gently lysed and separated into nuclear and cytoplasmic fractions by using a previously described fractionation technique (Simon and Malim 1996). Then, levels of HIV-1 late reverse transcription products in each fraction were analyzed by semi-quantitative PCR, as described above. Results revealed differential effects of C-terminal mutants on HIV-1 DNA nuclear import. In the wt, D64E and RK263,4AA virus-infected samples, there were respectively 70%, 72% and 68% of viral DNA associated with nuclear fractions (Fig.III.5C.(upper panel, lanes 1 and 2; 3 and 4; 9 and 10) and D). For KK240,4AE mutant, approximately 51% of viral DNA was nucleus-associated (Fig.III. 5C (upper panel, lane 7 and 8) and D). Remarkably, in KK215,9AA infected sample, viral cDNA was found

predominantly in the cytoplasm and only approximately 21% of viral DNA was associated with the nuclear fraction (Fig.III.5C (upper panel, lane 5 and 6) and D).

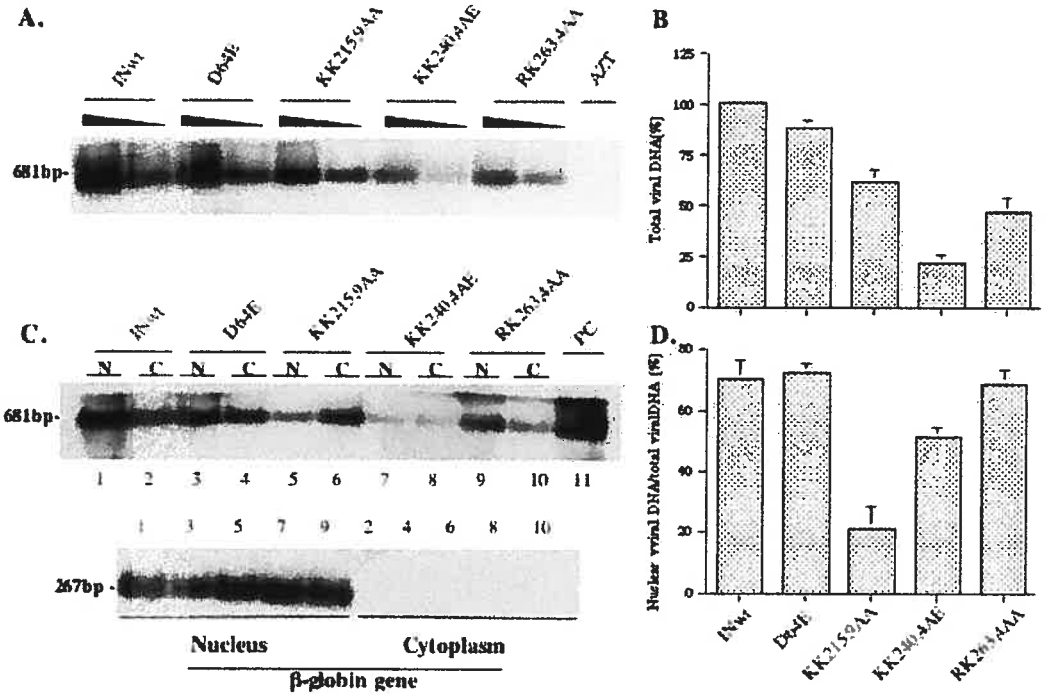


**Fig.III.5. Effects of different IN mutants on HIV-1 reverse transcription and DNA nuclear import.** Dividing C8166 T cells were infected with equal amounts of different HIV-1 IN mutant viruses. **A)** At 12 hours post-infection, $1x10^6$ cells were lysed and the total viral DNA was detected by PCR using HIV-1 LTR-Gag primers and Southern blot. **B)** Levels of HIV-1 late reverse transcription products detected in panel A were quantified by laser densitometry and viral DNA level of the wt virus was arbitrarily set as 100%. Means and standard deviations from two independent experiments are presented. **C)** At 24 hours post-infection, $2x10^6$ cells were fractionated into cytoplasmic and nuclear fractions as described in Materials and Methods. The amount of viral DNA in cytoplasmic and nuclear fractions were analyzed by PCR using HIV-1 LTR-Gag primers and Southern blot (upper panel, N. nuclear fraction; C. cytoplasmic fraction). Purity and DNA content of each subcellular fraction were monitored by PCR detection of human globin DNA and visualized by specific Southern blot (lower panel). D). The percentage of nucleus-associated viral DNA relative to the total amount of viral DNA for each mutant was also quantified by laser densitometry. Means and standard deviations from two independent experiments are shown.

Meanwhile, the integrity of fractionation procedure was validated by detection of β-globin DNA, which was found solely in the nucleus and levels of this nucleus-

associated cellular DNA were similar in each nuclear sample (Fig.III.5C, lower panel).

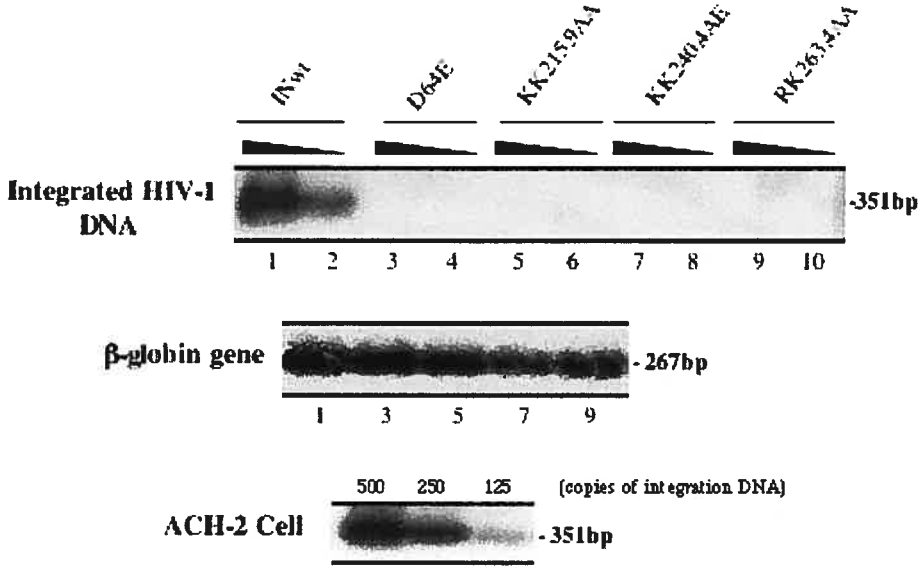


**Fig.III.6. Effect of IN mutants on HIV-1 proviral DNA integration**. Dividing C8166 T cells were infected with equal amounts of different HIV-1 IN mutant viruses. At 24 hours post-infection, $1x10^6$ cells were lysed and serial-diluted cell lysates were analyzed by two-step Alu-PCR and Southern blot for specific detection of integrated proviral DNA from infected cells (Upper panel). The DNA content of each lysis sample was also monitored by PCR detection of human β-globin DNA and visualized by specific Southern blot (middle panel). The serial-diluted ACH-2 cell lysates were analyzed for integrated viral DNA and as quantitative control (lower panel). The results are representative for two independent experiments

Even though the C-terminal mutants were shown to significantly affect HIV-1 reverse transcription and/or nuclear import, the various low levels of nucleus-associated viral DNA during the early stage of replication (Fig.III.5C) may still be accessible for viral DNA integration. To address this question, $1x10^6$ dividing C8166 T cells were infected with equivalent amounts of each single cycle replicating virus stock (5cpm/cell), as indicated in figure 6 and after 24 hours of infection, the virus integration level was checked by using a previously described sensitive Alu-PCR technique (Ao, Yao et al. 2004), Results revealed

that, while the wt virus resulted in an efficient viral DNA integration (Fig.III.6, upper panel; lanes 1 and 2), there was no viral DNA integration detected in D64E mutant (lanes 3 to 4) and in all three C-terminal mutant infection samples (lanes 5 to 10), although similar levels of cellular β-globin gene were detected in each sample (Fig.III.6, middle panel). These results suggest that, in addition to affecting HIV-1 reverse transcription and nuclear import, all three C-terminal IN mutants tested in this study also negatively affected viral DNA integration. Overall, all of these results indicate that all three IN C-terminal mutants are belonged to class II mutants, which affected different early steps during HIV-1 replication. Among these mutants, the KK240,4AE showed the most profound inhibition on reverse transcription and the KK215,9AA, and to a lesser extent, KK240,4AE, impaired viral DNA nuclear translocation during early HIV-1 infection in C8166 T cells.

## DISSCUTION

In this study, we performed mutagenic studies to analyze different regions in the C-terminal domain of HIV-1 IN that contribute to protein nuclear localization as well as their effects on virus infection. First, our analyses showed that specific lysine mutations introduced in two highly conserved tri-lysine regions in the C-terminal domain of HIV-1 IN impaired protein nuclear accumulation. Second, infection experiments revealed that all three C-terminal mutant viruses (KK215,9AA, KK240,4AE and RK263,4AA) exhibited more severe defect of induction of β-Gal positive cells and luc activity, as compared to an IN class 1 mutant D64E virus, in $CD4^+$ HeLa-β-Gal cells, dividing and non-dividing C8166 T cells. It suggests that all three C-terminal mutant virus infections may have defects at steps prior to integration. Further analysis of total viral DNA synthesis, viral DNA nuclear import and integration indicates that all three C-terminal mutants displayed a class II mutant profile. Even though all of

them reduced viral reverse transcription levels, the mutant KK240,4AE showed the most profound inhibitory effect. In addition, the mutant KK215,9AA, and to a lesser extent, KK240,4AE, impaired viral DNA nuclear translocation. These IN mutant-induced defects do not appear to result from various effects of mutants on Gag-Pol processing and maturation given that RT and IN were complemented *in trans* in this HIV-1 single-cycle infection system. Rather, the effect of different IN mutants on reverse transcription and viral DNA nuclear import is likely originated from a role of mutants within the maturing PIC complexes.

Previous work by Gallay *et al.,* have proposed an atypical bipartite NLS ($^{186}$KRK and $^{211}$KELQKQITK) in HIV-1 IN by finding that IN mutants K186Q and Q214/216L lost their karyophilic feature and their ability to bind to karyopherin α *in vitro* (Gallay, Hope et al. 1997). Even though these results were confirmed by Petit and colleagues by studying the intracellular localization of HIV-1 Flag-IN (Petit, Schwartz et al. 2000), other studies, using GFP-IN fusion protein, did not reveal the importance of K186Q and Q214/216L mutations for HIV-1 IN nuclear localization (Tsurutani, Kubo et al. 2000; Devroe, Engelman et al. 2003; Lu, Limon et al. 2004). Therefore, the definition of region(s) in HIV-1 IN contributing to the protein nuclear localization is still controversial. In this study, we investigated the intracellular localization of several IN-YFP fusion proteins including the C-terminal-deletion mutant $IN_{1\text{-}212}$-YFP, substitution mutants $IN_{KK215,9AA}$-YFP and $IN_{KK240,4AE}$-YFP and found that all of these IN fusion mutants impaired protein nuclear accumulation. It suggests that two C-terminal tri-lysine regions $^{211}$KELQKQITK and $^{236}$KGPAKLLWK contribute to IN nuclear localization. Interestingly, the study by Maertens *et al* also showed that the fusion of HIV-1 IN C-terminal fragment alone with GFP rendered fusion protein to be exclusively in the nucleus, speculating that the C-terminal domain may have a role in HIV-1 nuclear import (Maertens, Cherepanov et al. 2003). However, at this moment, we still could not exclude the possibility that the IN nuclear accumulation could be facilitated by the DNA binding ability of IN

protein, as suggested by Devroe *et al* (Devroe, Engelman et al. 2003). It has to be noted that two studies have previously observed the nuclear localization of GFP-IN fusion proteins although the C-terminal domain of IN was deleted from the fusion protein (Tsurutani, Kubo et al. 2000; Maertens, Cherepanov et al. 2003). It has also been shown that both N-terminal zinc binding domain and the central core domain of HIV-1 IN are involved in its interaction with a cellular protein, human lens epithelium-derived growth factor/transcription coactivator p75 (LEDGF/p75) and this IN/LEDGF/p75 interaction is required for GFP-IN nuclear localization (Maertens, Cherepanov et al. 2003). However, our deletion analysis by using IN-YFP fusion protein failed to reveal the importance of both N-terminal and core domains for IN nuclear localization (Fig.III.1). One explanation for this discrepancy could be different orientations of fusion proteins used in our study (IN-YFP) and other studies (GFP-IN). It is possible that different forms of fusion proteins may differentially affect the ability of IN to interact with LEDGF/p75 and consequently affect their ability for nuclear targeting. Therefore, it would be interesting to test whether $IN_{KK215,9AA}$-YFP and $IN_{KK240,4AE}$-YFP could loss their ability to interact with LEDGF/p75. These studies are underway.

An important question that needs to be addressed is the impact of nuclear localization-defective IN mutants on HIV-1 replication. Given that most IN mutants characterized so far are classified as class II mutants that cause pleiotropic damage including defects in viral morphogenesis, reverse transcription and integration (Nakajima, Lu et al. 2001; Lu, Limon et al. 2004), we used a previously described VSV-G pseudotyped HIV-1 RT/IN trans-complement single-cycle replication system (Wu, Liu et al. 1997; Ao, Yao et al. 2004) to minimize differential effects of IN mutants on virus maturation. Also, in our infection experiments, a specific integration-defective class I mutant D64E virus was introduced in order to monitor the viral gene expression from unintegrated HIV-1 DNA species that are already translocated into nucleus during virus

infection. It is known that certain levels of selected viral gene expression (*tat and nef*) from unintegrated viral DNA species are detected during this Class I mutant infection (Engelman, Englund et al. 1995; Wu and Marsh 2001; Brussel and Sonigo 2004). Interestingly, our infection analysis revealed that more profound infection defects were found for all three IN C-terminal mutant viruses KK215,9AA, KK240,4AE and RK263,4AA than D64E mutant virus in Hela-CD4-CCR5-β-Gal cells, dividing and non-dividing C8166 T cells (Fig.III. 3 and 4). These results suggest that these C-terminal IN mutants may affect early steps such as reverse transcription and/or nuclear import and consequently result in a reduced level of viral DNA in the nucleus, which is accessible for *tat* and *nef* expression, To understand the mechanism(s) underlying replication defects of each C-terminal mutant, levels of total reverse transcription were analyzed during early viral infection. Consistent with a previous study (Wu, Liu et al. 1999), infection with D64E mutant virus did not affect reverse transcription as compared to wt virus infection. However, all three C-terminal mutants display various levels of impaired HIV-1 reverse transcription (Fig.III.5A and B). The mutant KK240,4AE showed strongest inhibition of reverse transcription (22% compared to the wt level (100%)), while mutants KK215,9AA and RK263,4AA reached to 61% and 46% (Fig.III.5A and B). These data indicate that all of these IN mutants, especially KK240,4AA, negatively affect reverse transcription at early viral infection. Consistently, recent studies have shown that the C-terminal domain of IN contributes to efficient reverse transcription and this domain of IN was able to bind to heterodimeric RT (Wu, Liu et al. 1999; Hehl, Joshi et al. 2004; Zhu, Dobard et al. 2004). It is possible that these C-terminal mutants, especially for KK240,4AE, may disrupt the interaction between IN and RT and result in decreased viral cDNA synthesis.

Subsequently, we examined levels of nucleus- and cytoplasm-associated viral DNA during early virus infection. Results clearly show that the nuclear

localization defective mutant KK215,9AA leads to significantly reduced levels of viral DNA in the nucleus, as compared to the wt and D64E viruses (Fig.III. 5C and D). It suggests that the Q region is in fact important for HIV-1 nuclear import. Consistently, a recent study by Lu *et al* also observed that infection of K215A/K219A mutant induced more than 3-fold lower luc activity compared to class I IN mutant D64N/D116N (Lu, Limon et al. 2004). Moreover, similar to our experimental system, their study revealed that, in the context of VSV-G pseudotyped virus infection in Jurkat cells, 2-LTR circle DNA levels of K215A/K219A and Q214L/Q216L were significantly lower than other mutants V165A and C130G, even though the inhibition of viral reverse transcription mediated by these mutants were comparable (Lu, Limon et al. 2004). In addition, KK240,4AE mutant also showed a modest impairment of viral DNA nuclear import (Fig.III.5C and D). In fact, this mutant exhibited the most profound infection defect, compared to other two mutants (KK215,9AA and RK263,4AA) (Fig. 3 and 4). This may be due to combined effects of this mutant on both reverse transcription and viral DNA nuclear import, as shown in Fig.III.5. One interesting question is whether such profound infection defect of KK240,4AE mutant virus could be due to a structural alteration by replacing glutamic acid (E) for lysine at position of 244. It seems to be unlikely since 1) the effect of this mutant on nuclear import was not as dramatic as KK215,9AA mutant (as shown in Fig.III.5); 2) Wiskerchen *et al* have reported that infection of MAGI cells with two other IN mutants K236A/K240A and K244A/E246A mutants, that are located in the same region as our KK240,4AE mutant, resulted in 0 and 4 β-Gal positive cells, while infection of class I IN mutants produced 700 to 1400 β-Gal positive cells (Wiskerchen and Muesing 1995). All of these observations suggest that this region indeed plays an important role for IN activities during early stage of virus infection prior to integration. Also, it has to be noted that although similar inhibition of reverse transcription was seen for KK215,9AA and RK263,4AA mutants, RK263,4AA mutant induced two to three fold higher level of β-Gal

positive cells and luc activity than KK215,9AA mutant (Fig. III.3 and 4). This is expected since KK215,9AA affected both reverse transcription and nuclear import, while RK263,4AA mutant only impaired reverse transcription (Fig.III.5). In addition, our analysis could not detect viral DNA integration in each C-terminal mutant infection (Fig.III.6), even though they displayed various low levels of nucleus-associated viral DNA (Fig.III.5C). It suggests that these IN mutants may also negatively affect viral integration during their infection. Alternatively, it could be possible that these mutants may have additional defect(s) at an undefined postnuclear entry step that is required for viral DNA integration, as suggested by Lu *et al* (Lu, Limon et al. 2004). Consistently, their recent reports have shown that several IN mutants in same regions, including K215A/K219A, E244A and R262A/K264A, completely lost virus replication ability in CD4+ Jurkat T cells (Lu, Limon et al. 2004; Lu, Ghory et al. 2005).

Up to now, the mechanism(s) underlying the action of HIV-1 IN in viral PIC nuclear import is still unclear. Since IN is a component of viral PIC, at least two factors may affect the contribution of IN to viral PIC nuclear import: first, IN needs to directly or indirectly associate with viral DNA and/or other PIC-associated proteins in order to participate in driving viral DNA into the nucleus; second, IN needs to have a NLS and/or bind to other karyophilic proteins for nuclear translocation. Any mutation disrupting one of these two abilities would affect IN's action for viral DNA nuclear import. A recent study evaluated the effect of several IN core domain mutants targeting key residues for DNA recognition on HIV-1 replication and indicated that, while all of these IN mutants maintained their karyophilic properties, viruses harboring these mutants still severely impaired viral DNA nuclear import (Ikeda, Nishitsuji et al. 2004). In our study, both KK215,9AA and KK240,4AE mutants clearly lost their karyophilic properties and negatively affected viral DNA nuclear import. However, it is still premature to define these regions acting as IN NLS, even though a previously

described IN mutant Q214/216L, which is also located in proximal tri-lysine domain, has been shown to reduce IN-karyopherin α interaction *in vitro* (Gallay, Hope et al. 1997). More studies are required for further characterization of molecular mechanisms underlying the action of these IN mutants during HIV-1 DNA nuclear import.

# Chapter IV

## Interaction of Human Immunodeficiency Virus Type 1 Integrase with the Cellular Nuclear Import Receptor Importin 7 and its Impact on Viral Replication

## ABSTRACT

Similar with all other viruses, Human Immunodeficiency Virus Type 1 (HIV-1) depends heavily on cellular factors for its successful infection. In this study we have investigated the interaction of HIV-1 integrase (IN) with several nuclear import factors by using co-immunoprecipitation assay. Our results showed that IN interacts specifically with Imp7 in the cells, but not binds to importin 8 (Imp8) and importin α (Rch1). In contrast, another HIV-1 karyophilic protein MAp17, that was capable of binding Rch1, was unable to interact with Imp7, suggesting that these two HIV-1 proteins may interact with different cellular pathways during HIV-1 replication. Genetic analysis revealed that the C-terminal domain of IN is the region responsible for interaction between IN with Imp7, and an IN mutant (KK240,4AA/RK263,4AA) disrupted the Imp7-binding ability of the protein, suggesting that both regions ($^{235}$WKGPAKLLWKG and $^{262}$RRKAK) within the C-terminal domain of IN contributed to efficient IN/Imp7 interaction. Using a VSV-G pseudotyped HIV single-cycle replication system, we demonstrated that the IN/imp7 interaction deficient mutant completely inhibited the replication of HIV-1 and displayed impairment at both viral reverse transcription and nuclear import steps. Moreover, transient knockdown of Imp7 in both HIV-1 producing and target cells resulted in 2.5 to 3.5-fold inhibition of HIV infection. Altogether, our results indicate that HIV-1 IN specifically interacts with Imp7 and this viral/cellular protein interaction contributes to an efficient HIV-1 infection.

## INTRODUCTION

To carry out a successful infection, HIV-1 takes advantage of various host cellular proteins and cellular pathways. The interactions between cellular proteins and viral components take place during various steps of the HIV-1 life cycle including viral DNA nuclear import. The most striking feature of HIV-1 is its ability to replicate in nondividing cells, which depends on the capacity of the virus to transport its cDNA, as a part of a large preintegration complex (PIC), from the cytoplasm to the nucleus by an active and energy-dependent process (1-3). However, the mechanism by which the PIC translocates across the nuclear membrane into the nucleus of nondividing cells is still not fully understood. It has been shown that three HIV-1 PIC-associated proteins including MAp17, IN and Vpr possess karyophilic properties, and contribute to nuclear translocation of viral PICs into the nucleus. This action is accomplished through their interactions with karyophilic cellular proteins, thereby directing the PIC through the nuclear pore (4-10). In addition, a *cis*-acting element named the central DNA flap located in the 3' region of the *pol* gene sequence was also shown to contribute to HIV-1 nuclear import in both dividing and nondividing cells (11-14).

Nuclear import of proteins in mammalian cells can be mediated by several distinct pathways. Importin α/β heterodimer meditates nuclear import of protein harboring a classical nuclear localization signal (NLS), which either contains a cluster of basic amino acids or two basic clusters (bipartite NLS) (for reviews see (15,16)). Also, importin β (Impβ) was shown to bind to and import proteins, such as HIV-1 Tat, Rev and HTLV Rex, independently of the importin α (Impα) (17-21). Similarly, transportin, an impβ-related receptor, imports its substrates (hnRNP proteins) by directly binding to the glycine-rich M9 domain of the protein (22,23). Moreover, based on the similarity to Impβ, several other nuclear import receptors, including improtin 7 (Imp7) and importin 8 (Imp8), have also been identified (24).

Imp7 was one of several cellular importins that bind and mediate nuclear import of ribosomal proteins in mammalian cells and it was also found to translocate other proteins such as glucocorticoid receptor and histone H1 into the nucleus (18,25-27). In the case of histone H1, Jakel *et al* have demonstrated that two receptors, Impβ and Imp7 form a heterodimer, and this complex is a functional unit required for the nuclear import of histone H1. Imp 8 has also shown to contribute to the nuclear import of signal recognition particle protein 19 (28). However, whether and how these cellular proteins contribute to the nuclear import of HIV-1 PIC during the early stage of viral infection remains to be defined.

HIV-1 integrase (IN) is a 32-kDa protein that plays a key role in viral cDNA integration into the host chromosome. In addition, this viral protein has also been shown to contribute to other steps during the early stage of HIV-1 replication, including reverse transcription (29,30) and viral DNA nuclear import (7,10,31,32). Even though IN has been well documented to possess karyophilic properties (7,10,33-35), the mechanism by which HIV-1 IN contributes to the nuclear import of the viral PIC is still not fully understood. Some previous studies have showed that IN is capable of binding to Impα in *in vitro* binding assays (7,36,37), but the *in vitro* nuclear import assay results concerning whether Impα plays a role in the nuclear translocation of IN and/or HIV-1 DNA is still controversial (33,37,38). A cellular component, human lens epithelium-derived growth factor/transcription coactivator p75 (LEDGF/p75) was initially implicated in contributing to the nuclear translocation of HIV-1 (39,40). However, the following study came to the conclusion that the interaction of IN with LEDGF/p75 may not be required for IN nuclear localization (41). Several studies have further identified that LEDGF/p75 is important for tethering IN as well as the viral PIC to chromosomal DNA and contributes to controlling the location of HIV DNA integration (41-43). In attempts to search for other cellular factor(s) involved in HIV-1 nuclear import, Fassati *et al* have reported that Imp7 contributes to HIV-1 PIC nuclear import through an *in vitro* nuclear import assay. Their study also showed that small interfering RNA (siRNA)

mediated Imp7-knockdown inhibited HIV-1 replication (38). In addition, their *in vitro* binding assay showed that recombinant IN could pull down several cellular nuclear import receptors including Impα, Impβ, Imp7 and transportin from HeLa cell lysates, suggesting that the action of Imp7 for HIV-1 PIC nuclear import may be through its binding to HIV-1 IN (Supplementary materials in (38)). However, a recent study by Zielske *et al.*, did not reveal the impact of Imp7 knockdown on HIV-1 and SIV nuclear import in macrophages (44). Therefore, the functional role of Imp7 action during HIV-1 replication remains to be defined.

In this study, we have investigated the interaction of HIV-1 IN with several cellular importins by using a cell-based co-immunoprecipitation assay. Our results indicate that HIV-1 IN, specifically interacts with Imp7, but not with Impα (Rch1) and Imp8, and this IN/Imp7 interaction takes place in cells. We also showed that another HIV-1 karyophilic protein MAp17, that is capable of binding Rch1, was unable to interact with Imp7. In addition, our mutagenic analysis demonstrated that two regions ($^{235}$WKGPAKLLWKG and $^{262}$RRKAK) in the C-terminal domain of IN are critical for its Imp7-binding ability. In attempt to elucidate the contribution of the IN/Imp7 interaction to HIV-1 replication, our results indicate that an Imp7-binding defective IN mutant virus lost infectivity and displayed defects during both reverse transcription and nuclear import. Moreover, our experiments revealed that HIV-1 produced from Imp7-depleted cells exhibited 2.5 to 3.5-fold reduced infection in Imp7-knockdown susceptible cells.

## MATERIAL AND METHODS

**Construction of different viral and cellular protein expressors**-To generate CMV-YFP-IN fusion expressor, the full-length wild-type HIV-1 IN cDNA was amplified from HIV-1 HxBru provirus (45) by polymerase chain reaction (PCR) using 5'-*Bgl*II primer (5'-GCCAGATCTTTCTTAGATGGAATAGATAAG-3') and 3'-*BamH*I primer (5'-CTAAACGGATCCATGTTCTAA-3'). The amplified HIV-1 IN fragment was cloned in frame to 3' end of EYFP cDNA in a pEYFP-C1 vector (BD Biosciences Clontech). The CMV-IN-YFP and CMV-$IN_{50-288}$-YFP expressors used in the study were previously described (32). To construct different CMV-YFP-IN deletion mutants, cDNA fragments encoding aa 1-212 and1-240 of IN were generated by PCR with 5'-*Bgl*II primer and 3 primers (5-CAATTCCCGGGTTTGTATGTCTGTTTGC-3; 5-CCAGACCCGGGTTGCTGGTCCTTTCCA-3) and was inserted into pEYFP-C1 vector at *Bgl*II and *Xma*I sites. Different IN substitution mutants were generated by a two-step PCR-based method (46) by using 5'-*Bgl*II primer, 3'-*XhoI* primer and complementary primers containing desired mutations. The amplified IN cDNAs harboring specific mutations were then cloned into pEYFP-C1 vector. To generate HIV-1 provirus NL*A*.3-BruΔBgl/Luc, the sequence from *ApaI* to *SalI* site (nt 1556 to nt 5329, +1= start of NL*A*.3 initiation of transcription) in a RT/IN/Env defective HIV-1 provirus NLlucΔBgl/ΔRI (32) was replaced by the corresponding sequences of HIV-1 provirus HxBru (45). The genotype of this molecular clone is 5' LTR *gag*$^+$ *pol*$^+$ *vif*$^+$ *vpr*$^+$ *tat*$^+$ *rev*$^+$ *vpu*$^+$ *env*$^-$ *nef*$^-$ 3' LTR.

The pGEX-Imp7 and pGEX-IImp8 plasmids encoding for Xenopus Imp7 and human Imp8 cDNAs were generously provided by Dr. Yamamoto (26) and used as PCR templates for constructing CMV-T7-Imp7, CMV-T7-Imp8 plasmids The cDNA encoding Rch-1 was amplified from a pET-21-Rch1. The amplified Imp7, Imp8 and Rch-1 fragments were digested with *BamH*I and *Not*I and cloned at 3' end of T7-tag in a SVCMV-T7 vector. The MAp17G2A cDNA was generated by

PCR from HIV-1 provirus HxBru using primers (5' primer: 5-ATAGCTAGCGAGATGGCTGCGAGA-3 ; 3' primer : 5-CTGCGGATCCGGGTAATTTTGGCTGAC-3) and the second amino acid glycine was changed to alanine. Then, the CMV-MAG2A-YFP was constructed by inserting a HIV-1 MAG2A cDNA in frame at 5' of YFP cDNA in the CMV-YFP-N1 plasmid (BD Biosciences Clontech). All newly-contructed expressing plasmids were subsequently analyzed by DNA sequencing to confirm the sequence and the presence of mutation and/or deletions.

**Antibodies and chemicals-**Antibodies used in immunoprecipitation or western blot are as follows. The purified rabbit anti-GFP polyclonal antibody, mouse monoclonal anti-GFP antibodies were obtained from Molecular Probes Inc. The mouse anti-T7 antibody was obtained from Novagen Inc (Darmstsdt, Germany). The rabbit anti-human Imp7 antibody was kindly provided by Dr. A. Fassati (38). The rabbit anti-IN antibodies (Cat No. 757) and the purified recombinant HIV-1NL4.3 IN protein (Cat No. 9420) were obtained through AIDS Research Reference Reagent Program, Division of AIDS, NIAID, NIH. The human anti-HIV serum was kindly provided by Dr. Eric A. Cohen and described previously (45). The ECL$^{TM}$ HRP-conjugated donkey anti-rabbit IgG and the sheep anti-mouse IgG were purchased from Amersham Biosciences. The western blot detection ECL kit was purchased from PerkinElmer Life Science (Boston, MA). CHAPS (3-((3-cholamidopropyl)-dimethylammonio)-1-propane-sulfonate) was purchased from Sigma Chemical Co.

**Cell culture and transfection-**Human embryonic kidney 293T cells and HeLa-β-gal-CD4/CCR5 cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin and streptomycin. The $CD4^+$ C8166 cells were maintained in RPMI-1640 medium containing 10% FCS and antibiotics. DNA transfection in 293T cells was performed with standard calcium phosphate DNA precipitation method. After 48 hours of transfection, cells were harvested and ready for different experiments.

**IN/Imp7 binding assays using immunoprecipitation (IP) and western blot (WB)**-To test protein expression and the protein-protein interaction in mammalian cells, 293T cells were transfected or co-transfected with corresponding protein expression plasmids. After 48 h of transfection, cells were lysed with a CHAPS lysis buffer (199 medium containing 0.5% CHAPS and a protease inhibitor cocktail (Roche)) on ice for 30 min and clarified by centrifugation at 13,000 rpm for 30 min at 4°C. Then, the supernatant was subjected to IP with rabbit anti-GFP or the corresponding antibody. Immunoprecipitats were resolved by 10% SDS-PAGE gel followed by western blot using mouse anti-T7 or mouse anti-GFP antibodies, respectively. Also, the total T7-tagged protein expression in cell lysates was sequentially immunoprecipitated with mouse anti-T7 antibody followed by western blot using the same antibody.

To test the interaction of HIV-1 IN with endogenous Imp7, 293T cells were mock-transfected or transfected YFP or IN-YFP expression plasmids and the same IP and WB protocols were used as described above, except using rabbit anti-Imp7 antibody to check the bound endogenous Imp7. Meanwhile, non-transfected 293T cell lysate was loaded directly in SDS-PAGE gel as positive control.

**In Vitro Binding Studies** - To produce GST, GST-Imp7 proteins, the *E. coli* BL21 cells transformed with pGEX-4T-GST or pGEX-4T-GST-Imp7 plasmids were cultured in LB medium (0.1 mg/ml ampicillin). Protein expression was induced by adding isopropyl-1-β-D-thiogalactopyranoside (1 mM) for 3 h at 37 °C. Bacteria were harvested, suspended in 35 ml of ice-cold column buffer, and broken by sonication (five 30-s pulses at 100 watts, Sonics & Materials, Inc.). The resulting lysates were centrifuged for 30 min at 13000 rpm and pass through a glutathione-sepharose 4B column (Amerham Pharmacia Biotech Inc). After being washing by column buffer, the bound GST and GST-Imp7 proteins were eluted by glutathione buffer (100mM reduced gluthathione (Roche), 120mM NaCl, 100mM Tris-HCl pH 8.5). Finally, the eluted protein was dialyzed in PBS to remove high concentration of glutathione.

For *in vitro* binding experiments, the equal amounts of recombinant GST or GST-Imp7 protein were incubated with a recombinant HIV-1 IN in 199 medium containing 0.1% CHAPS, for 2 hours at 4 °C. Then, 100 µl of glutathione-sepharose 4B beads were added and incubated for additional one hour. The beads were washed and the bound proteins were eluted with 50 mM glutathione, loaded onto a 12.5% SDS-PAGE for western blot analysis with rabbit anti-IN antibodies.

**Transient knockdown of Imp7 in 293T and HeLa-β-gal-CD4/CCR5 cells** - 293T cells and HeLa-β-gal-CD4/CCR5 cells were plated at $2x10^5$ cells/well in 6-well plates and transfected at the next day with 100 pmol of Imp7-specific small interfering RNA (siRNA) duplex (IPO7-HSS116173) with Lipofectamine™ RNAiMAX Reagent (Invitrogen). After 18 h of first transfection, another Imp7 siRNA duplex (IPO7-HSS116174) was transfected again into cells. These two Imp7-siRNA duplexes (Stealth RNAi), IPO7-HSS116173 and IPO7-HSS116174, were synthesized by Invitrogen Inc and the targeting sequence are respectively corresponding to Imp7 mRNA nucleotides 1990-2013 (5'-UAAGCAGAUUCCCUCAAGCUGUUGG-3'), and to Imp7 mRNA nucleotides 610-633 (sense 5'-AAUGCUGCAUUGCUGGCUACCAAUGG-3'). In parallel, transfection of a scramble RNA (sc-RNA) (purchased from Santa Cruz Biotechnology) was used as control. After 48 and 72 hours post-transfection, cells were used for different HIV-1 provirus transfection and virus infection, respectively.

**Virus production and infection** – To test the effect of Imp7-binding defective mutant on HIV replication, a vesicular stomatitis virus G (VSV-G) glycoprotein pseudotyped single-cycle replicating virus were produced in 293T cells, as described previously (32). Briefly, 293T cells were transfected with a RT/IN defective HIV-1 provirus NLlucΔBglΔRI, each CMV-Vpr-RT-IN (wt/mutant) expressor and a VSV-G expressor. Introduction of IN mutations into CMV-Vpr-RT-IN expressor was through a PCR-based method as described previously (32). To produce viruses from Imp7-siRNA- or sc-RNA-transfected

cells, the same amount of cells transfected with imp7 siRNA or scramble RNA were re-plated at 36 h after the first transfection, and after additional 12 h, cells were transfected with different HIV-1 proviruses. After 48 hours of provirus transfection, viruses were collected from the supernatant through an ultracentrifugation, and virus titers were quantified by using HIV-1 p24 Antigen Capture Assay Kit (purchased from The NCI-Frederick AIDS Vaccine Program).

To infect CD4+ C8166 T cells, equal amounts of viruses (adjusted by amount of virion-associated p24) were incubated with C8166 T cells at $37^0$C for 4 h. At different time points post-infection, $1x10^6$ cells from each sample were collected, lysed with 50 μl of luciferase lysis buffer (Fisher Scientific Inc). 10 μl of cell lysate was subjected to the luciferase assay by using a TopCount®NXT™ Microplate Scintillation &Luminescence Counter (Packard, Meriden) and the luciferase activity was valued as relative luciferase units (RLU). Each sample was analyzed in duplicate and the average deviation was calculated. To test the effect of Imp7 knockdown on HIV-1 infection, at 72 h after being transfected with Imp7-siRNA or sc-RNA, HeLa-β-gal-CD4/CCR5 cells were infected with equal amounts of different viruses in the presence of DEAE-Dextran (20 μg/ml). At 48h post-infection, the HIV-1 infection were monitored by measurement of the luc activity level and/or the amount of β-Gal positive cells, as described previously (47).

**HIV-1 reverse-transcribed and nuclear imported DNA detection by PCR and Southern blotting -** C8166 T cells were infected with equal amount of the VSV-G pseudotyped INwt or mutant viruses for 2 h, washed with PBS and cultured in RPMI medium. At 12 or 24 hours post-infection, equal number ($1x10^6$ cells) of cells were collected and processed for detecting total viral DNA synthesis or nucleus- and cytoplasm-associated viral DNA by PCR and southern blotting, as described previously (32).

## RESULTS

### 1. HIV-1 IN interacts with Imp7, but not with Imp8

To investigate the interaction of HIV-1 IN with different cellular nuclear import factors, we first tested the interaction of HIV-1 IN with cellular nuclear import receptors Imp7 and Imp8, by using a cell-based co-immunoprecipitation (co-IP) assay. SVCMV-T7-Imp7 and -Imp8 expressing plasmids were constructed by inserting Imp7 and Imp8 cDNAs into a SVCMV-T7 vector at the 3' end of a T7 tag encoding sequence (Fig.IV.1A), as described in experimental procedures. Also, a previously described HIV-1 IN-YFP fusion protein expressor CMV-IN-YFP (32) and a CMV-YFP expressor were used in the study and showed in figure 1A. First, the expressions of these proteins were checked by transfecting each of these plasmids into 293T cells, and processed using anti-GFP or anti-T7 immmunoprecipitation (IP), followed by western blot with corresponding antibodies. Results showed that IN-YFP and YFP were detected at positions 58 and 27 kDa respectively (Fig.IV.1B, lanes 2 and 3), while T7-Imp7 and T7-Imp8 were at positions that ranged between 110 to 130 kDa (Fig.IV.1B, lanes 4 and 5).

To test whether IN-YFP could bind to different importins, the YFP or IN-YFP expressor was co-transfected with each importin expressor in 293T cells, as indicated in Fig.1C. After 48 h, cells were lysed with CHAPS lysis buffer (199 medium containing 0.5% CHAPS), and immunoprecipitated using rabbit anti-GFP antibody. Precipitated complexes were run on an SDS-PAGE, followed by western blot with anti-T7 antibody (Fig.IV.1C, upper panel). Interestingly, results revealed that, while YFP protein did not co-precipitate with any importin (Fig.IV.1C, upper panel, lane 1, 2), the IP of IN-YFP specifically co-pulled down T7-Imp7 (Fig.IV.1C,

Lane 3), but not T7-Imp8 (Fig.IV.1C, lanes 4). Meanwhile, the immunoprecipitated

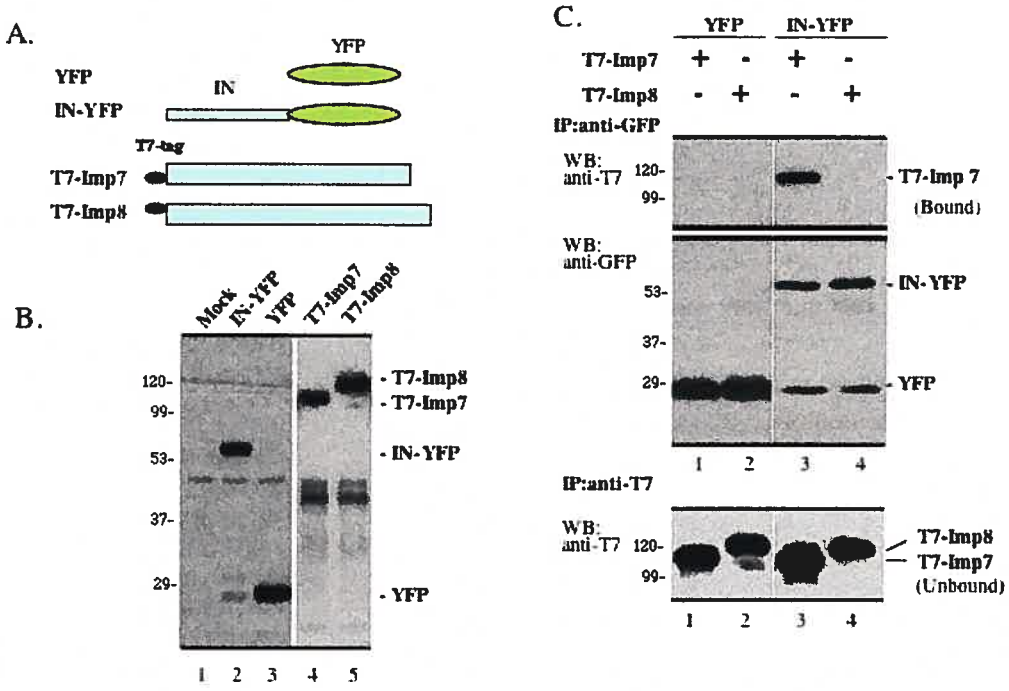


**Fig. IV.1. Interaction of HIV-1 IN and importin 7.** A) Schematic representation of constructs of IN-YFP, T7-Imp7 and -Imp8. For IN-YFP, a full-length wild-type HIV-1 IN was fused in frame to the N-terminus of EYFP. For T7-Imp7 and Imp8, a T7-tag (9 amino acids) was fused in frame to the N-terminus of Imp7 and Imp8. B) Expression of IN-YFP and T7-Imp7 and T7-Imp8. Cell lysates from about $6x10^5$ 293T cells transfected with CMV-YFP, CMV-IN-YFP or indicated importin expressors were analyzed by immunoprecipitation (IP) with rabbit anti-GFP antibody followed by western blotting using mouse anti-GFP antibody (lanes 1 to 3) or IP with mouse anti-T7 antibody followed by western blotting using the same antibody (lanes 4 and 5). C) The *in vivo* co-IP assay. CMV-IN-YFP was co-transfected with plasmid T7-Imp7 (lane 3) or T7-Imp8 (lane 4) into $2x10^6$ 293T cells. As a control, CMV-YFP also was co-transfected with each importin expressing plasmid (lanes 1 and 2). After 48 h of transfection, cells were lysed by 0.5% CHAPS buffer and immunoprecipitated with rabbit anti-GFP antibody. The immunoprecipitated complexes were resolved by 12.5% SDS-PAGE and immunoblotted with either mouse anti-T7 antibody (upper panel) or mouse anti-GFP antibody (middle panel). The unbound T7-Imp7 and T7-Imp8 were also checked by sequential IP with anti-T7 antibody followed by immunoblotting with the same antibody (lower panel).

IN-YFP and YFP in each sample respectively were checked by anti-GFP western blot, and similar levels of each protein were detected (Fig.IV.1C; middle panel, lanes 3, 4). To rule out the possibility that the co-precipitated T7-Imp7 was due to differential levels of importin expression in each transfection sample, the cell lysates were processed using sequential IP with anti-T7 antibody followed by anti-T7 Western blot, and the results showed similar expression levels of each importin in different samples (Fig.IV.1C; lower panel). All of these results indicated that IN specifically interacts with Imp7, but not with Imp8.

**2. HIV-1 IN interacts with Imp7 in the cells**

The next question we asked was whether the IN/Imp7 interaction occurs in the cells or after cells had been lysed. To address this question, IN-YFP or T7-Imp7 expresser was individually transfected into different 293T cell cultures, as indicated in figure 2A. After 48 hours, cells from two transfected cultures were mixed, lysed with 0.5% CHAPS lysis buffer and incubated in 4°C for two hours. Then, the presence of IN/Imp7 interaction in the cell lysate was checked by anti-GFP IP, followed by anti-T7 western blot. In parallel, cells co-transfected with both IN-YFP and T7-Imp7 expressers were mixed with the same amounts of mock-transfected cells and processed identically. Strikingly, the co-precipitated T7-Imp7 was only detected in co-transfected cell lysate, but not in mixed cell lysate from individually transfected cell samples (Fig.IV.2A, upper panel, compare lane 2 with 3). These results clearly indicate that the interaction of IN-YFP and T7-Imp7 takes place in the cells. Again, the specific detection of IN/Imp7 complex in co-transfected cells, was not due to the varying levels of expression of IN-YFP or T7-Imp7 protein in the different samples (Fig.IV.2 A, middle panel and lower panel; lanes 2 and 3). To further test the interaction between IN-YFP and endogenous Imp7, 293T cells were transfected with CMV-YFP or CMV-IN-YFP expressor, lysed by 0.5% CHAPS

lysis buffer and immunoprecipitated with anti-GFP. The co-precipitated endogenous Imp7 was checked by western blot with a rabbit anti-human Imp7

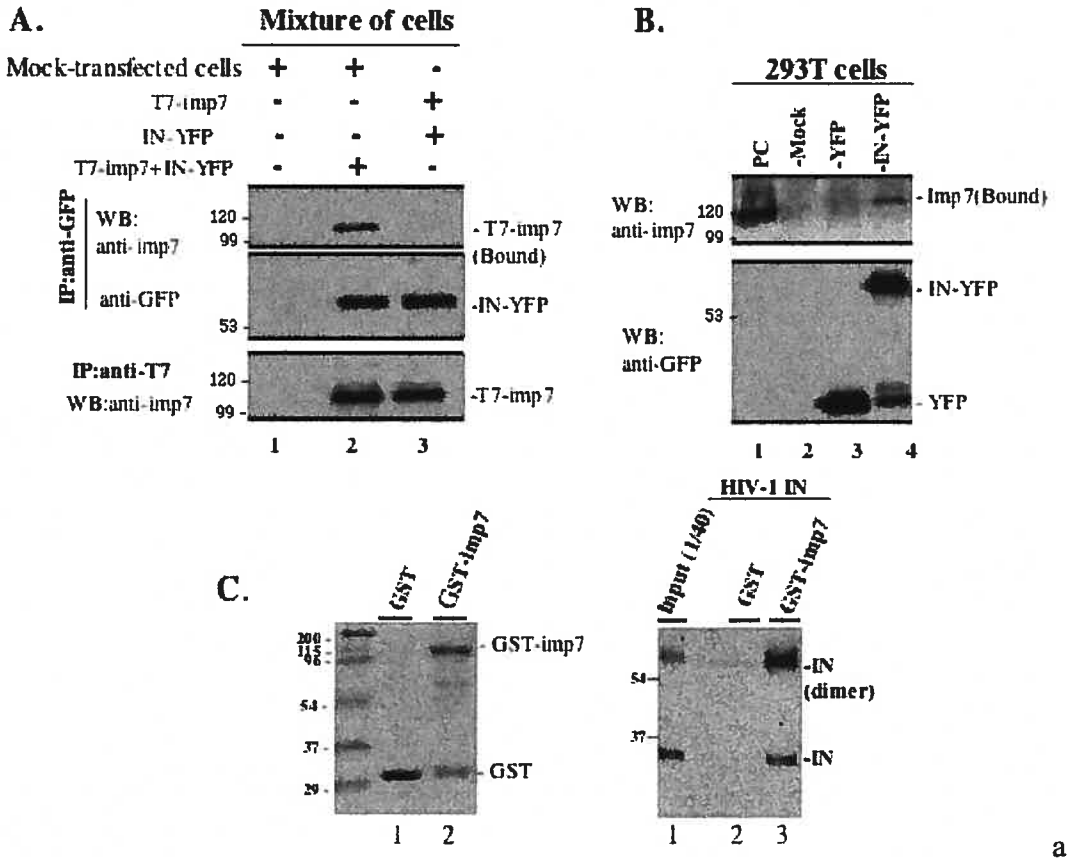


**Fig.IV. *2.* HIV-1 IN interacts with endogenous Imp7 and the IN-Imp7 interaction takes place in the cells and *in vitro*.** A) IN-Imp7 interaction in the cells. The IN-YFP and T7-Imp7 plasmids were co-transfected (lane2) or transfected individually (lane3) into 293T cells. After 48 h, cells were mixed accordingly, lysed and analyzed with co-IP using the same procedure as Fig.1C. Upper panel: co-precipitated Imp7; Middle panel: The expression of IN-YFP; Lower panel: the unbound Imp7. B) IN interacts with endogenous Imp7. $10x10^6$ 293T cells were mock-transfected (lane 1) or transfected with CMV-YFP (lane 2) and CMV-IN-YFP (lane 3). After 48 h of transfection, cells were lysed and analyzed by co-IP Using the same procedure as Fig.1.C. In parallel, $0.5x10^6$ of non-transfected 293T cells were lysed with the same lysis buffer and loaded in SDS-PAGE as positive control (PC). Upper panel: the endogenous Imp7 and co-precipitated endogenous Imp7; Middle panel: The expression of YFP and IN-YFP. C) *In vitro* interaction between IN and Imp7. Left panel: GST (lane 1) and GST-Imp7 (lane 2) were expressed in *E coli* and affinity-purified from glutathione-sepharose 4B colume and shown by the Coomassie Blue staining. Right panel: Equal amounts of GST (lane 2) and GST-

Imp7 (lane 3) were incubated with a purified recombinant HIV-1 IN followed by GST-pull down and analyzed on SDS-PAGE gel by western blot with rabbit anti-IN antibodies. The bound IN, in both of dimmer and monomer forms were indicated at the right side of the photograph.

antibody. Meanwhile, the non-transfected 293T cell lysates were directly loaded into SDS-PAGE as the positive control (Fig.IV.2B, lane 1). We found that IN-YFP, but not YFP, was able to pull down the endogenous Imp7 (Fig.IV.2B, upper panel, compare lane 4 to lane 3), indicating that IN-YFP interacts with endogenous Imp7 in 293T cells.

The following question that needed to be addressed was whether IN binding to Imp7 could be through a direct protein interaction. We produced the purified recombinant GST and GST-Imp7 proteins in an *E coli* expression system, and the purified protein in each sample was tested by directly loading protein samples in an SDS-PAGE, and verified by Coomassie Blue staining of the gel (Fig.IV.2C, left panel) and by western blot with specific anti-Imp7 antibody (data not shown). To test the direct interaction of IN and Imp7 *in vitro*, similar amounts of purified GST and GST-Imp7 were incubated with a purified recombinant HIV-1 IN in 199 medium containing 0.1% CHAPS for 2 h at 4 °C, followed by an additional one hour incubation with glutathione-sepharose 4B beads. Then, the bound protein complex was eluted out with 10 mM glutathione, and loaded onto a 12.5% SDS-PAGE gel, followed by western blot analysis with anti-IN antibodies. Results showed that the purified HIV-1 IN, in both of dimmer and monomer forms, was able to specifically interact with GST-Imp7, and not with GST (Fig.IV.2C, right panel). Thus, the binding of IN to Imp7 may be through a direct protein/protein interaction.

### 4. Differential binding ability of HIV-1 IN and MAp17 to impα (Rch1) and Imp7

The importin α/β nuclear translocation pathway has been implicated in assisting with HIV-1 nuclear import (6,7). Several HIV-1 proteins, including MAp17, Vpr

and IN have been shown to be able to interact with Impα in *in vitro* binding assays (6,7,36,38,48). In this study, we attempted to test whether HIV-1 IN could interact with Rch1, a member of the human importin α family (49), by using co-IP assay. A T7-tagged Rch1 expressing plasmid (CMV-T7-Rch1), and an HIV-1 $MAp17_{G2A}$ mutant-YFP fusion protein expressing plasmid (CMV-$MA_{G2A}$-YFP) were constructed. In $MAp17_{G2A}$-YFP, the second amino acid glycine in MAp17 protein was replaced by alanine, and this MAp17 mutant was previously shown to capable of binding to Rch1 in a cell-based co-IP system (6). After IN-YFP or $MA_{G2A}$-YFP were co-expressed with T7-Rch1 in 293T cells, their interaction with Rch1 was analyzed using the same co-IP and western blot protocols, as described in figure 1. Consistent with previous report (6), $MA_{G2A}$-YFP was shown to be able to bind to T7-Rch1 (Fig.IV.3A; lane 4). However, IN-YFP did not show any interaction with T7-Rch1 (Fig.IV.3A, lane 3). In contrast, while T7-Imp7 co-precipitated with IN-YFP, no T7-Imp7 was detected in the immunoprecipitated $MA_{G2A}$-YFP sample (Fig.IV.3B, compare lane 4 to 3). These results suggest that HIV-1 IN and MAp17 may interact with different cellular nuclear import factors during HIV-1 replication.

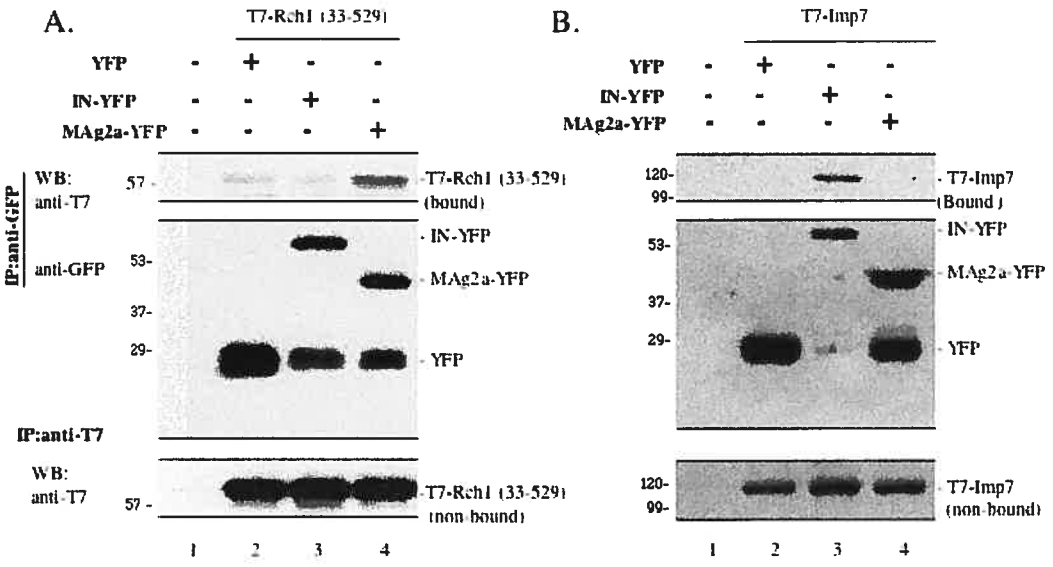


**Fig. IV.3. Differential binding ability of HIV-1 MAp17 and IN to cellular importins Rch1 and Imp7.** A) HIV-1 $MAp17_{G2A}$, but not IN, binds to T7-Rch1. 293T cells were co-transfected by CMV-T7-Rch1 with YFP (lane 2), IN-YFP (lane

3) or MAp17$_{G2A}$-YFP expressor (lane 4) and followed by the co-IP assay. Upper panel shows the co-precipitated T7-Rch1; Middle panel: the expression of YFP, IN-YFP or MAp17$_{G2A}$-YFP; Lower panel: the unbound T7-Rch1. B) HIV-1 IN, but not MAp17$_{G2A}$, binds to Imp7. 293T cells were co-transfected with YFP (lane2), IN-YFP (lane3) or MAp17$_{G2A}$-YFP (lane4) plasmid and T7-Imp7 expressor and followed by the co-IP assay. Upper panel shows the co-precipitated T7-Imp7; Middle panel: the expression of YFP, IN-YFP or MAp17$_{G2A}$-YFP; Lower panel: the unbound T7-Imp7.

5. **Delineation of region(s) of HIV-1 IN required for its interaction with Imp7**

To delineate which region(s) within HIV-1 IN is required for its Imp7-binding, we first tested a previously described IN N-terminal deletion mutant (CMV-IN$_{50-288}$-YFP) expressor (32) (Fig.IV.4A) for Imp7-binding. The co-IP analysis revealed that, similar to the IN-YFP, the IN$_{50-288}$-YFP bound efficiently to T7-Imp7 as well (Fig.IV.4B, compare lane 5 to lane 4), indicating that the N-terminal domain of IN is not required for the IN/Imp7 interaction.

To test the core domain and the C-terminal domain of IN for their contribution towards Imp7-binding, we constructed three YFP-IN expressors, including CMV-YFP-INwt and two IN C-terminal deletion mutants (CMV-YFP-IN1-212 and CMV-YFP-IN1-240) (Fig.IV.4A). With the CMV-YFP-INwt expressor, the PCR-amplified HIV-1 IN full length cDNA, was placed in frame at the 3' end of the YFP cDNA, while for CMV-YFP-IN1-212 and CMV-YFP-IN1-240, sequences encoding for the last 76 and 48 aa of IN was removed respectively. Expression of each YFP-IN fusion protein along with its ability to bind Imp7 was tested in 293T cells by co-transfecting each YFP-IN fusion protein expressor with the T7-Imp7 plasmid. The YFP-INwt, YFP-IN1-212 and CMV-YFP-IN1-240 fusion proteins were detected at molecular weights ranging approximately from 47 to 58 kDa (Fig.IV.4C, middle panel, lanes 3 to 5). Interestingly, the co-IP experiments revealed that while YFP-INwt efficiently bound to T7-Imp7, two IN C-terminal deletion mutants were unable to bind to T7-Imp7 (Fig.IV.4C, upper panel, compare lane 3 to lanes 4 and 5),

suggesting that the C-terminal region encompassing residues 240 and 288 is required for IN interacting with Imp7.

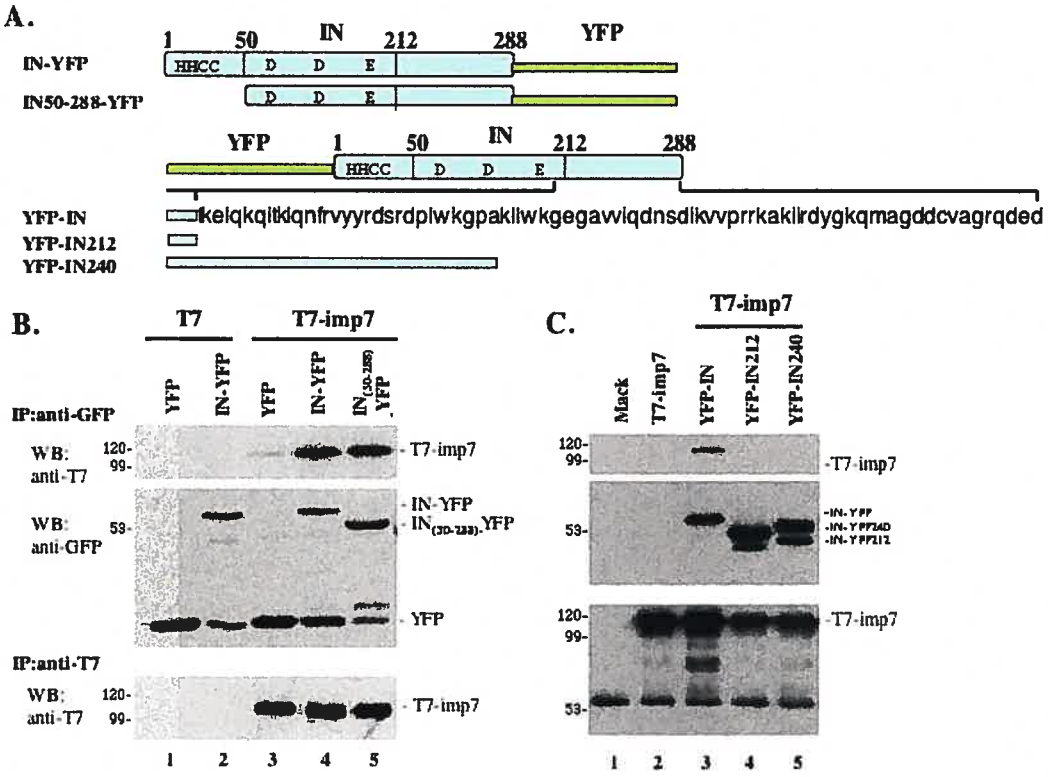


**Fig.IV.4. Deletion analysis for the necessary region(s) of HIV-1 IN for its interaction with Imp7**. A) Schematic representation of IN-YFP and YFP-IN truncated proteins used for binding assay. B) The N-terminal domain is dispensable for IN-Imp7 interaction. The YFP (lane 3), IN-YFP (lane 4) and $IN_{50-288}$-YFP (lane 5) were co-expressed with T7-Imp7 in 293T cells. In parallel, the YFP and IN-YFP were cotransfected with T7-expressor as negative control (lanes 1 and 2). Upper panel: the co-precipitated T7-Imp7; Middle panel: the expression of YFP, IN-YFP and $IN_{50-288}$-YFP. Lower panel: the unbound T7-Imp7. C) The C-terminal domain is required for IN-Imp7 interaction. The YFP-IN full–length protein (lane 3), YFP-IN1-212 (lane 4), YFP-IN1-240 (lane 5) were co-transfected with T7-Imp7 expressor in 293T cells and their Imp7-binding was analyzed by using the same protocol as described in figure 1C. Upper panel: co-precipitated T7-Imp7. Middle panel: Expression of YFP, YFP-IN and YFP-IN mutants. Lower panel: unbound T7-Imp7.

**6. Critical amino acids required for efficient IN/Imp7 interaction**

To further identify the amino acids in the IN C-terminal region required for Imp7-binding, several IN mutants in the form of YFP-IN fusion proteins were constructed (Fig.IV.5A). Mutants YFP-IN240,4AA, YFP-IN263,4AA and YFP-INKKRK were designed to target a tri-lysine region ($^{235}$WKGPA$^{240}$KLLW$^{244}$KG), and/or an arginine/lysine rich region ($^{262}$RRKAK). Previous studies have implicated that these tri-lysine and arginine/lysine rich regions are involved with efficient HIV-1 reverse transcription, viral DNA nuclear import and/or integration (32,34). The YFP-IN249, 50AA and YFP-IN258A were constructed to target highly conserved residues valine and lysine at positions 249, 250 and 258 (Fig.IV.5A). An IN core domain mutant YFP-INKR186,7AA was also included in this study, since it was previously implicated in assisting HIV-1 nuclear import (7). Each YFP-IN mutant plasmid was co-transfected with the T7-Imp7 expressor in 293T cells, and processed by the co-IP assay to test each protein's Imp7-binding ability. Results revealed that while other IN mutants did not affect the ability to bind Imp7 (Fig.IV. 5B, lanes 4, 5, 10), the YFP-IN263,4AA mutant significantly impaired the ability of IN to bind Imp7, and the YFP-INKKRK mutant was unable to interact with imp7 (Fig.IV.5B, lanes 9 and 10). Thus, all these results indicate that both tri-lysine region ($^{235}$WKGPA$^{240}$KLLW$^{244}$KG) and the arginine/lysine rich region ($^{262}$RRKAK) is required for efficient interaction between IN and Imp7.

***6.* Effect of Imp7-binding defective IN mutant on HIV-1 infection in CD4+ C8166 T cells**

Given that IN mutant INKKRK lost its Imp7-binding ability, we next examined the effect of this IN mutant on HIV-1 replication. This mutant was introduced into a previously described VSV-G pseudotyped HIV-1 single-cycle replication system (13,32). Briefly, the INKKRK mutant was first introduced into a CMV-Vpr-RT-IN expressor. Then, the VSV-G pseudotyped HIV-1 single cycle replicating virus (vKKRK) was produced in 293T cells by co-transfection with CMV-Vpr-RT-

INKKRK, an RT/IN-deleted HIV provirus NLlucΔBgl/ΔRI and a VSV-G

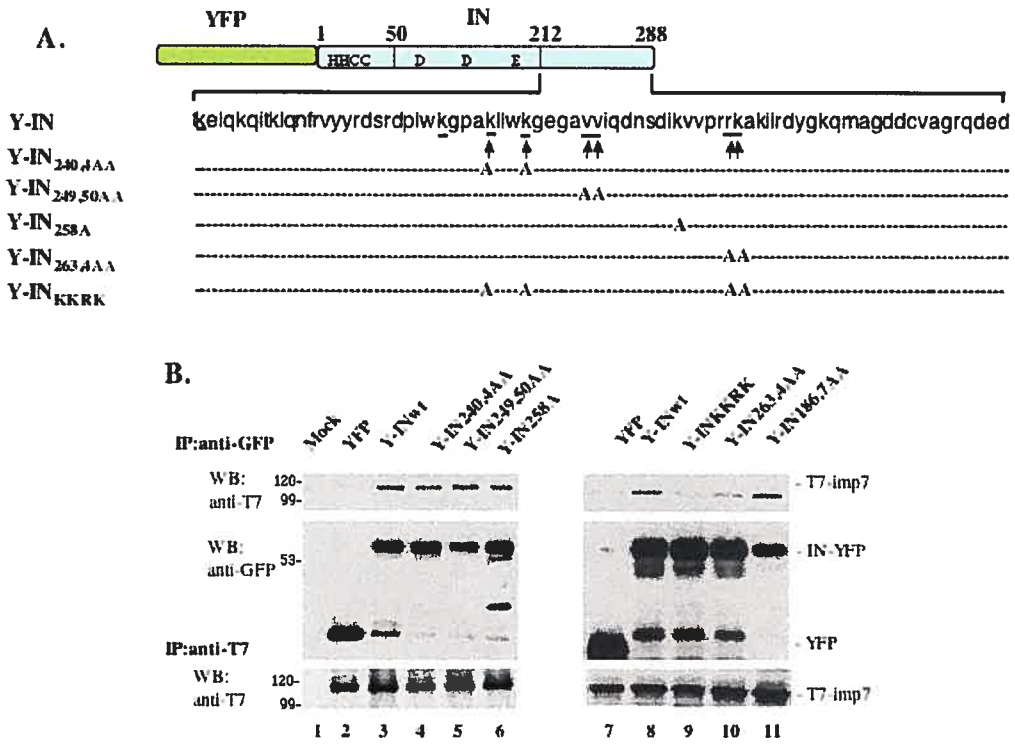


**Fig. IV. 5. The critical amino acid in the C-terminal domain of IN required for efficient IN/Imp7 interaction.** A) Diagram of HIV-1 IN domain structure and introduced mutations at the C-terminal domain of the protein. The positions of introduced mutation are shown at the bottom of sequence. B) Requirement of amino acids in the C-terminal domain of IN for efficient IN/Imp7 interaction. The YFP (lanes 2 and 7), YFP-INwt (lanes 3 and 8) and different YFP-IN mutant expressors were co-transfected with T7-Imp7 expressor in 293T cells and after 48 h of infection, cells were lysed with CHAPS lysis buffer and the IN/Imp7 interaction for each IN mutant was analyzed by using the same protocol as described in figure 1C. Upper panel: co-precipitated T7-Imp7. Middle panel: Expression of YFP, YFP-INwt and YFP-IN mutants. Lower panel: unbound T7-Imp7. The position of each immunoprecipitated and co-precipitated proteins were indicated on the right side of the gel.

expressor, as described previously (32). In parallel, the VSV-G pseudotyped wild type virus (vINwt) and IN class I mutant D64E virus (vD64E), were also produced in parallel as controls. After each virus stock was harvested, the trans-incorporation

of RT and IN as well as the Gag composition in the viral particle was analyzed using

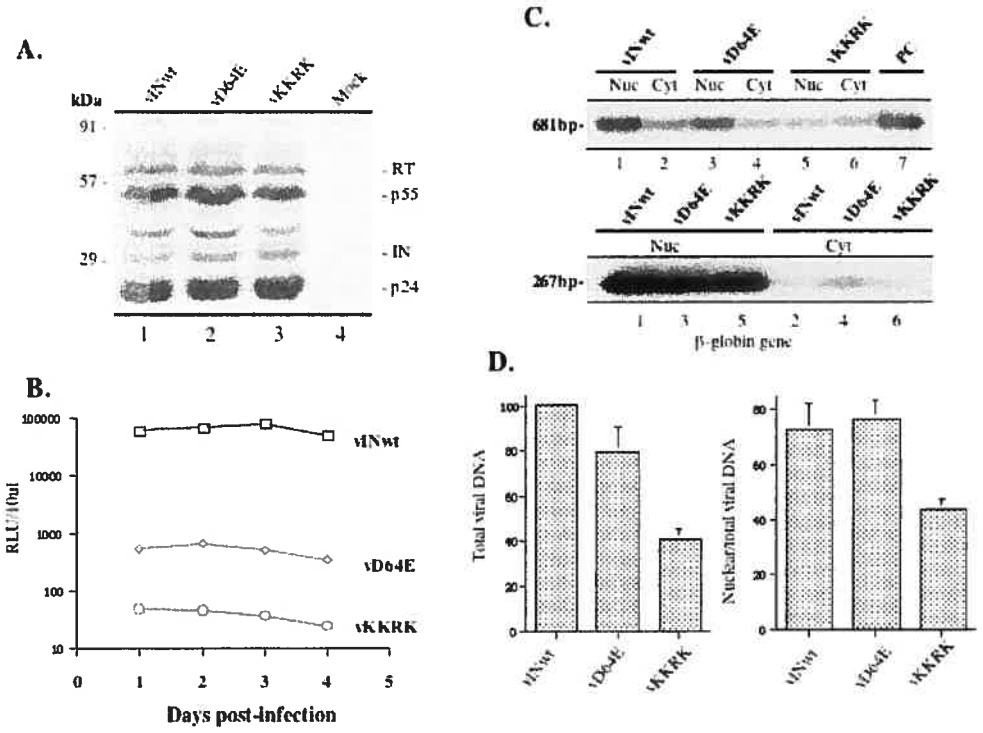


**Fig.IV.6. Imp7-binding defective IN mutations disrupted HIV-1 single-cycle replication and affected both reverse transcription and viral nuclear import.** A) 293T cells were transfected with a RT, IN and Env deleted HIV-1 provirus NLlucΔBglΔRI with different Vpr-RT-IN (wt/mutant) expressors and a VSV-G expressor. The produced virus particles (lane 1 to 3) were lysed and directly loaded in 12% SDS-PAGE and analyzed by Western blot with human anti-HIV serum. The positions of HIV-1 Gag, RT and IN proteins are indicated. B) The $CD4^+$ C8166 cells were infected with the wt, vD64E or vKKRK viruses. At different time intervals after infection, the equal amount ($1x10^6$) of cells was collected and cell-associated luciferase activity was measured by luciferase assay. C) Effect of Imp7-binding defect mutant on HIV-1 reverse transcription and DNA nuclear import. At 24 h post-infection, $2x10^6$ C8166 cells were gently lysed and fractionated into the cytoplasmic and the nuclear fractions. The amount of viral DNA in both fractions were analyzed by PCR using HIV-1 LTR-Gag primers and Southern blot. Nuc. nuclear fraction; Cyt. cytoplasmic fraction, The purity and DNA content of each subcellular fraction were monitored by PCR detection of human globin DNA and visualized by specific Southern blot (lower panel). D) The total amounts of viral DNA (left panel) and the percentage of nucleus-associated viral DNA relative to the total amount of viral DNA (right panel) for each infection sample was also quantified by laser densitometry. Means and standard deviations from two independent experiments are shown.

western blot with a human anti-HIV positive serum. Results showed that similar amounts of RT, IN and Gagp24 were detected in each virus preparation (Fig.IV.6A). Then, equal amount of each virus stock (as adjusted by amounts of HIV-1 Gagp24) was used to infect $CD4^+$ C8166 cells. At different time intervals, the luciferase (luc) activity in equal amounts of cells was measured, as shown in figure 6B. Since D64E mutant virus (vD64E) is unable to mediate viral DNA integration, its infection expressed very low luc activity, which only reached 0.8% of the luc activity level detected from the wt virus infection (Fig.IV.6B). Interestingly, the luc activity detected from the vKKRK virus infection was considerably lower than that of the D64E mutant virus at different time points (Fig.IV.6B), indicating that the vKKRK virus lost its replication ability in CD4+ C8166 cells.

To test at which step the Imp7-binding defect mutant virus infection was affected, the cytoplasm- and nucleus-associated viral DNA levels were analyzed at 24 hours post-infection, using semi-quantitative PCR and southern blot. For the vKKRK virus infection, the level of total viral DNA (including the cytoplasm- and nucleus-associated viral DNA levels) was reduced by approximately 60%, compared to the total viral DNA level detected from the wt virus infection (Fig.IV.6C, upper panel, compare lanes 5 and 6 to lanes 1 and 2, and D, left panel). Moreover, results indicated that for the wt and vD64E virus infections, approximately 73 and 77% of viral DNA were associated with nuclear fractions (Fig.IV.6C (upper panel, lanes 1 to 4) and D, right panel). However, during vKKRK infection, only 44% of viral DNA was nucleus-associated (Fig.IV.6C (upper panel, lanes 5 and 6) and D, right panel). The integrity of the fractionation procedure was also validated by detection of β-globin DNA, which was found solely in the nucleus, and levels of this cellular DNA were similar in each nuclear sample (Fig.IV.6C, lower panel). Taken together, all of these results indicate that the Imp7-binding defect mutant virus vKKRK was unable to replicate in C8166 cells and displayed impairment at both viral reverse transcription and nuclear import.

**7. Effect of Imp7-knockdown on HIV-1 replication**

To further elucidate the contribution of Imp7 to HIV-1 replication, we also investigated the effect of small interfering RNA (siRNA)-mediated Imp7-knockdown on HIV-1 replication. First, we tested the efficiency of Imp7 knockdown, the Imp7-siRNA (100 pmol) was introduced into 293T and HeLa-β-Gal-CD4/CCR5 cells once a day for two days (Fig.IV.7A) and at different time intervals, equal amounts of cells (0.5 $x10^6$ cells) were collected and monitored for Imp7 expression. Western blot results revealed that Imp7 protein expression were progressively decreased over the course of the experiments. At 48 hours following the first Imp7-siRNA transfection, the Imp7 protein level was reduced to approximately 30%, and at 96 hours, the level of Imp7 expression was reduced to <10% in both 293T and HeLa-β-Gal-CD4/CCR5 cells (Fig.IV.7B).

Next, we tested the effect of Imp7 knockdown on HIV-1 infection. To avoid the possibility that Imp7 might have effect on the late stage of viral replication and/or be packaged into viral particles and thus playing a role in subsequent viral infection, we first produced a VSV-G pseudotyped HIV-1 (NL4.3-BruΔBgl/luc+) from Imp7-siRNA- or scramble RNA (sc-RNA)-transfected 293T cells. The Imp7 protein expression in siRNA transfection cells was to < 10% (at 96 hours of siRNA transfection) when the viruses were collected. Then, viruses (si-virus and sc-virus) produced from Im7-siRNA- or scRNA-transfected 293T cells were normalized by HIV Gagp24 levels and used to infect siRNA-treated and sc-RNA-treated HeLa-β-Gal-CD4/CCR5 cells (target cells) (Fig.IV.7A). Results in figure 7C showed that there were no significant luc activity differences detected in sc-RNA- and si-RNA-treated target cells after being infected with sc-virus (Fig.IV.7C, bars 1 and 2) or in the sc-RNA-treated cells being infected by si-virus (FigIV.7C, bar 3). However, when siRNA-treated HeLa-β-Gal-CD4/CCR5 cells were infected with si-virus, the

luc activity was reduced to approximately 37% of the wt infection level (Fig.IV.7C, compare bar 4 to bar 1).

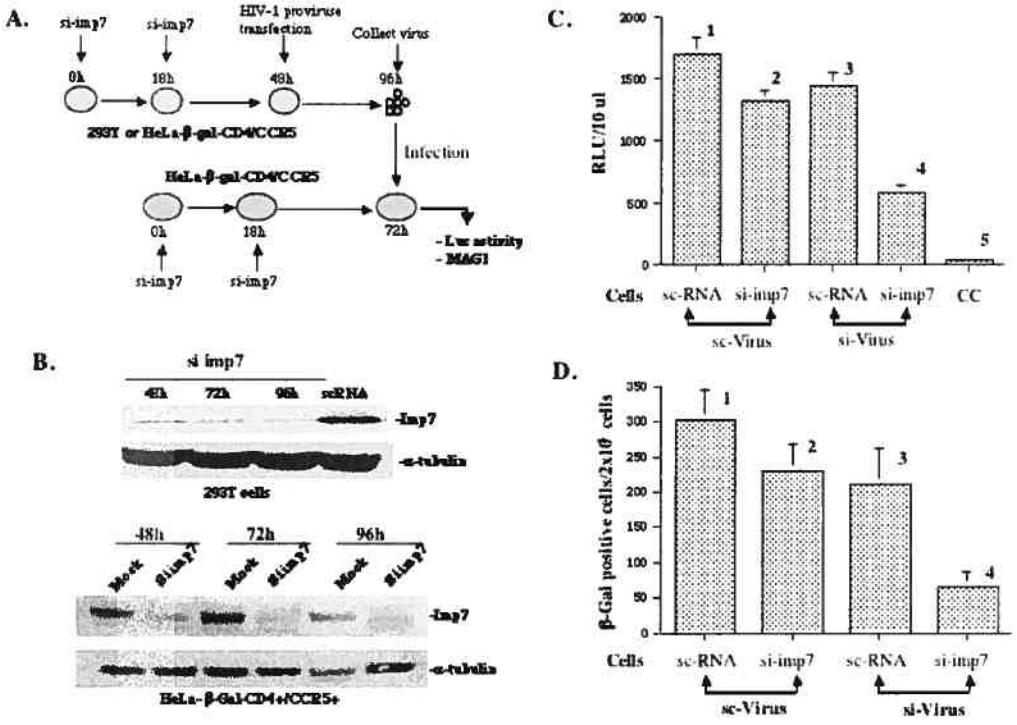


**Fig. 7. SiRNA-mediated silencing of Imp7 inhibits HIV-1 infection.** A) Experimental design for the duration of si-RNA treatments and HIV-1 transfection and infection. B) siRNA-mediated silencing of Imp7 in 293T and HeLa-β-Gal-CD4/CCR5 cells. Cells were transfected with 20 nM of siRNA at 0 and 18 hours. After 48, 72 and 96 hours post initial transfection, the Imp7 expression levels in each cell line was verified by Western blot with anti-Imp7 antibody (upper panel). Meanwhile, the expression of α-tubulin was also verified (lower panel). C) 293Tcells were treated with sc-RNA or si-imp7 once a day for two days and used to produced VSV-G-pseudotyped HIV-1 viruses containing luciferase gene (sc-virus and si-virus). Both viruses were then used to infect HeLa-β-gal-CD4/CCR5 cells that have been treated with Imp7-siRNA or scramble RNA for 72h. After 48h postinfection, the luciferase activity was measured. The results are representative for three independent experiments. D) sc-RNA or si-imp7 treated HeLa-β-Gal-CD4/CCR5 cells were infected with the wt HxBru virus produced from sc-RNA- or si-imp7-treated HeLa cells. After 48h postinfection, viral Infection was evaluated by MAGI assay. The results are representative for two independent experiments.

These observations were further extended to HIV-1 envelop-mediated viral infection. HIV-1 envelope competent si-HxBru and sc-HxBru viruses were produced in Imp7-siRNA and sc-RNA-treated HeLa-β-Gal-CD4/CCR5 cells by transfecting with a HIV-1 HxBru provirus (45) and used to infect the Imp7-siRNA and sc-RNA-treated HeLa-β-Gal-CD4/CCR5 cells at 72 h post-transfection. The numbers of β-Gal positive cells were evaluated by MAGI assay at 48h post infection. As expected, when Imp7-siRNA-treated HeLa-β-Gal-CD4/CCR5 cells were infected with si-virus, the β-Gal positive cell level was significantly reduced to approximately 27% of the wild type infection level (FigIV.7D, compare bar 4 to bar 1). Whereas, the β-Gal positive cell levels for sc-virus infection in siRNA-treated cells and for si-RNA virus infection in the control cells were slightly decreased to 76% and 70% of the wt infection level (Fig.IV.D, compare bars 2 and 3 to bar 1). All of these results indicate that the knockdown of Imp7 in both HIV-1 producing and target cells impaired HIV-1 infection.

## DISCUSSION

HIV-1 IN is a key enzymatic molecule which has been shown to contribute to different steps during the early stage of HIV-1 replication, including reverse transcription, viral DNA nuclear import and integration. Even though the exact mechanisms underlying the action of IN during each of these critical early steps is not fully understood, accumulative evidence indicates that IN is capable of interacting with different viral and cellular proteins at various steps during HIV-1 replication. This viral protein is well documented to possess karyophilic properties, and mutagenic analysis has revealed that some IN mutants significantly affect HIV-1 nuclear import (7,10,31-34,50). Several studies have showed that IN is capable of

binding to Impα and/or Imp7 in *in vitro* binding assays, suggesting that HIV-1 IN may recruit these cellular nuclear import factors during HIV-1 nuclear import (7,36,37). However, whether these cellular factors contribute to HIV-1 DNA nuclear import and replication still remains controversial (33,37,38,44). In this study, we have used a cell-based co-IP approach to investigate the interaction occurring between HIV-1 IN and several cellular nuclear import factors. Our results clearly show that HIV-1 IN, in both IN-YFP and YFP-IN fusion protein forms, specifically interacts with Imp7, but is unable to bind to Imp8 and Impα (Rch1). This specific IN/Imp7 interaction was further confirmed by using tandem affinity purification-tagged IN (TAP-IN) (data not shown). To rule out the possibility that the IN/Imp7 interaction could be an artifact of overexpression of these proteins in cells, the IN-YFP was transfected alone in 293T cells and co-IP results demonstrated that the endogenous Imp7 also was co-precipitated with IN-YFP, but not with YFP alone. Furthermore, our *in vitro* binding experiments revealed that the purified GST-Imp7 was able to pull down purified recombinant HIV-1 IN in both dimmer and monomer forms. Thus, all of these studies provide evidence that HIV-1 IN specifically interacts with Imp7.

Another HIV-1 karyophilic protein MAp17 was also implicated in HIV-1 nuclear import. However, unlike IN which was shown to be required for HIV-1 nuclear import in both dividing and nondividing cells, MAp17 contributes to HIV-1 nuclear import mainly in non-dividing cells (see reviews in (2,51). It implicates that these two viral proteins may utilize different nuclear import pathways during viral replication. In this study, we have compared the binding ability of these two HIV-1 proteins to Imp7 and Impα (Rch1). Interestingly, in contrast to IN, the MAp17 was unable to interact with Imp7 (Fig.IV.3). On the other hand, while IN failed to bind to Impα (Rch1), MAp17 was shown to interact with Impα, which conformed the previous observation by Gallay et al., showing that HIV-1 MAp17 bound to Rch1 in a co-IP experimental approach (6). These observations suggest that HIV-1 IN and

MAp17 may interact with different cellular machineries during HIV nuclear import and/or replication. Whether these viral/cellular protein interactions may synergize to assist with the HIV-1 replication, especially in non-dividing cells, remains an interesting question to be addressed.

Our deletion analysis identified that the Imp7-binding site(s) lies in the C-terminal domain of IN. The function of the C-terminal domain of IN was originally ascribed to that of nonspecific DNA binding, leading to suggestion that this domain may contribute to chromosomal DNA recognition during viral DNA integration (52-54). In addition, several recent studies indicate that the C-terminal domain of IN contribute to multiple steps during the early stage of HIV-1 replication, including reverse transcription, nuclear import and/or the postnuclear entry step(s) (30,32,34,55,56). In this study, two regions $^{235}$WKGPAKLLWKG and $^{262}$RRKAK within the C-terminal domain of IN were identified to contribute to the IN/Imp7 interaction. To investigate the effect of the IN/Imp7 interaction on HIV-1 replication, a VSV-G pseudotyped HIV-1 virus (vKKRK) containing the Imp7-binding defect IN mutant was produced. Infection analysis revealed that the vKKRK virus induced even lower luc activity than that of the integration-defective class I mutant D64E virus, indicating that this virus is replication defective. Further analysis showed that the Imp7-binding defective virus displayed impairments at both viral reverse transcription and nuclear import (Fig.IV.6C and D). Since this virus was shown to be non-infectious in C8166 cells (Fig.IV.6B), it is expected that this mutant virus also affect virus integration. Consistently, previous studies has already shown that several IN mutants targeting these positively charged residues inhibited HIV-1 integration (32,55). Given that most IN class II mutants cause pleiotropic damage during viral replication (55-57), we could not conclude that these defects were solely resulted from the lose of the IN/imp7 interaction. However, it is conceivable that the IN/imp7 interaction may have contributed to these critical steps during HIV-1 replication.

Another approach to validate the functional role of the IN/Imp7 interaction in HIV-1 replication is to directly target Imp7 expression within susceptible cells. Fassati *et al* previously showed that siRNA-mediated knockdown of endogenous Imp7 inhibited HIV infection (38). However, a recent study by Zielske *et al.,* did not reveal an inhibitory effect of Imp7 knockdown on HIV-1 nuclear import (44). It is worth to note that these studies were mainly focused on the wild type HIV-1 infection in Imp7-knockdown susceptible cells. It could not rule out the possibility that Imp7 might have effect on late stage of virus replication and/or be packaged into viral particles and playing a role in subsequent viral infection. Indeed, the study by Zielske *et al.,* observed a slight decrease of 2-LTR formation in Imp7-depleted target cells infected with viruses produced from a single-dose si-Imp7 treated cells in which Imp7 mRNA level was reduced to 77% at the time of virus collection (44). In this study, we have compared infections in si-RNA- or sc-RNA–depleted cells with viruses, which were produced from either siRNA- or sc-RNA-depleted 293T cells. Interestingly, results showed that depletion of Imp7 in both HIV-1 producing and target cells lead to 2.5 to 3.5-fold decrease of HIV-1 infection, as measured by either HIV-1-induced luc activity and the amount of β-Gal positive cells (Fig.IV.7C and D). However, such reduced HIV-1 replication was not observed for the infection of imp7-depleted cells with normal virus or the infection of normal cells with viruses produced from Imp7-depleted cells. All these results indicate that Imp7 contributes to an efficient HIV-1 replication. However, at this moment, the mechanism underlying the inhibitory effect of imp7 knockdown on viral replication still remains to be defined and more detailed studies are currently under way to address this question.

It should also be noted that the Imp7 knockdown in producer-target cell combination system only induced 2.5 to 3.5-fold reduction of viral infection. This result leads us to consider several possibilities. It could be possible that HIV-1 IN may have the ability to interact with multiple cellular nuclear import factors, and dissociation of one of them could not abolish HIV-1 replication. Similarly, it was

shown that the cellular ribosomal protein and the glucocorticoid receptor both utilize Imp7 and Impα/Impβ as its nuclear import receptors (18,26). Another possibility could be that IN interacts with a nuclear import receptor complex, in which Imp7 may act as an accessory cofactor. Indeed, previous studies have demonstrated that Imp7 is capable of forming a heterodimer with Impβ, and this heterodimeric complex has a higher binding affinity for histone H1 (25,27). Interestingly, it was also shown that the Impβ/RanGTP interaction appears to be essential for histone H1 import, while Ran-binding of Imp7 is dispensable (27). In addition, the limitation of siRNA knockdown technology used for this particular study should also be under consideration, since Imp7- siRNA treatment could not erase residual levels of Imp7 from cells, and such low amount of Imp7 may still be recruited by IN to support a lower level HIV-1 infection. Therefore, a genetic knock-out cell line will be required to address the impact of Imp7 on HIV-1 infection, as is being proposed by Vandegraaff et al., for the role of LEDGF/p75 in HIV-1 replication (58). Nevertheless, several lines of evidence from this study implicate the participation of Imp7 during HIV-1 infection and importantly, the identification of imp7-binding regions in the C-terminal domain of HIV-1 IN may provide opportunity for us to disrupt this viral/cellular protein interaction and consequently attenuate HIV-1 infection. Also, more detailed studies should be carried out to fully understand how this cellular nuclear import receptor contributes to efficient HIV-1 replication.

# Chapter V

# GENERAL DISCUSSION AND FUTURE DIRECTION

One key step in HIV-1 replication is the translocation of the viral genome from the cytoplasm to the nucleus to integrate viral DNA into the host cell chromosomes to form provirus. Oncoretroviruses require mitosis for the viral integration machinery to access the host cell DNA (Roe, Reynolds et al. 1993). In contrast, lentiviruses such as HIV-1 are able to productively infect non-dividing cells, including macrophages or quiescent T lymphocytes since its PIC has a mitosis-independent nuclear import capability (Bukrinsky, I.N. et al. 1992; Lewis 1992; Lewis and Emerman 1994; Bukrinsky and Haffar 1998; Bukrinsky and Haffar 1998; de Noronha, Sherman et al. 2001; Le Rouzic, Mousnier et al. 2002); (Weinberg 1991; Bukrinsky, I.N. et al. 1992). This has been shown to be important for viral persistence and pathogenesis in infected host, since the infection of post mitotic cells is essential not only for viral transmission and dissemination, but also for the establishment of persistent viral reservoirs (Meltzer, Skillman. D.R. et al. 1990; Innocenti, Ottmann et al. 1992; Ho, Cherukuri et al. 1994).

HIV-1 PIC, composed of viral RNA/DNA associated with viral proteins MA, NC, RT, IN, Vpr as well as cellular proteins, displays a diameter of 56nm. That greatly exceeds the 25nm central channel of nuclear pore complexes (NPC). Therefore, HIV PIC must traverse the NPC by active, energy-dependent import mechanism rather than passive diffusion. This complex contains the information that is necessary for nuclear localization and the enzymatic machinery required for integration. Recent studies have shown that three HIV-1 proteins, MA, Vpr and IN, may contribute to HIV-1 DNA nuclear localization and also revealed nuclear import signals in these viral gene products. In addition, a HIV-1 DNA structure determinant, the central DNA flap, has also been implicated to

contribute to efficient nuclear targeting of HIV-1 PIC. More interestingly, several studies have shown that HIV-1 IN and the DNA flap play important role in both dividing and non-dividing cells during HIV-1 nuclear import.

The HIV-1 central DNA flap is an unusual DNA structure, which corresponds to a triple-stranded intermediate created during the reverse transcription. Zennou and colleague revealed that the central DNA flap is necessary for HIV-1 replication in both dividing and nondividing cells presumably because it acts as a nuclear import signal for the PIC (Zennou, Petit et al. 2000). However, two other later studies showed that cPPT mutant viruses were still able to efficiently replicate, casting doubt on the importance of the central DNA flap in HIV-1 nuclear import (Dvorin, Bell et al. 2002; Limon, Nakajima et al. 2002). According to our study, by using a RT-IN trans-complement single cycle replication system, we confirmed the importance of the central DNA flap by showing that it is necessary and sufficient for efficient HIV-1 replication in dividing and nondividing cells. Our results also indicated that the central DNA flap enhances the establishment of HIV-1 infection in single-round replication assays by primarily facilitating nuclear import of proviral DNA. Different from the data reported by Zennou and Dvorin et. al., we found that the central DNA flap was important but not essential for viral infection of dividing and nondividing cells, which are consistent with the findings of several previous studies that showed that the central DNA flap conferred an infection advantage of 2- to 10-fold on VSV-G pseudotyped single-round HIV-1 vectors. However, the mechanism by which the central DNA flap contributes to the transport of HIV-1 PIC through the NPC remains unclear. It was proposed that the DNA flap might induce viral DNA to adopt a conformation that permit or facilitate the viral DNA transport through the nuclear pores. But recently, a study showed that the position of the DNA flap is not essential for its function in the context of HIV-1-derived lentiviral vector (De Rijck, Van Maele et al. 2005). It is also possible that

the DNA flap formation is a signal that can initiate the total structure changes of PIC, which allow this big complex to more efficiently cross the nuclear envelope. Alternatively, the DNA flap may be involved in nuclear import by interacting with nuclear import receptor as well as nucleoporins (Nups) or associating with viral protein(s) of the PIC to support this process.

In our study, we developed a RT/IN gene-deleted (2139) HIV-1 vector, which can efficiently transduce in $CD4^+$ cells upon trans-complementation with Vpr-RT-IN fusion proteins. Using this system, we can analyze the functional role of *cis-acting* elements in RT/IN gene regions, such as cPPT and CTS, without affecting their enzymematic function. It also allows us to introduce different IN mutations into Vpr-RT-IN expressor without differentially affecting viral morphogenesis and the activity of the central DNA Flap. Moreover, the large deletion in RT and IN sequence ensures that this HIV-1 vector is able to spread and replicate while at the same time minimizes the possibility of generating replication-compent virus by recombination. Further optimization of this HIV-1 single-cycle replication system will provide valuable tools to evaluate precisely viral dynamics in animal model and to develop vaccine approach against HIV-1 infection.

IN appears to be another important player for HIV-1 PIC nuclear import. It is a key protein for viral DNA integration and has karyophilic property. It was found that the nuclear import function of IN was essential for the productive HIV-1 infection of both dividing and non-dividing cells (Bouyac-Bertoia, Dvorin et al. 2001; Katz, Greger et al. 2003), which suggest that nuclear entry of HIV-1 PIC during mitosis may not be a passive process. However, questions have been raised concerning the molecular mechanism surrounding the karyophilic property of IN. Even though a non-classical bipartite NLS ($^{186}$KRK and $^{211}$KELQKQITK) has been proposed for IN (Gallay, Hope et al. 1997), follow-up studies could not prove the importance of this bipartite NLS since some mutants in these two regions still localized in the nucleus and/or did not play a role in viral nuclear

import (Petit, Schwartz et al. 2000; Tsurutani, Kubo et al. 2000; Bouyac-Bertoia, Dvorin et al. 2001; Lu, Limon et al. 2004). Moreover, some *in vitro* data suggested that nuclear localization of IN did not involved members of the karyopherin family of nuclear import receptors and was independent of GTP hydrolysis and Ran (Depienne, Mousnier et al. 2001). It was also proposed that IN nuclear localization might result from its ability to bind DNA (Devroe, Engelman et al. 2003). Hence, more studies are required to elucidate the exact functional role and mechanism of IN in HIV-1 PIC nuclear import.

In an attempt to define the essential region(s) in IN for protein nuclear localization, we have performed mutagenic analysis to study the intracellular localization of several IN-YFP fusion proteins including the C-terminal-deletion mutant $IN_{1\text{-}212}$-YFP, substitution mutants $IN_{KK215,9AA}$-YFP, $IN_{KK240,4AE}$-YFP and $IN_{RK263,4AA}$-YFP. We found that lysine residues in two regions ($^{211}$KELQKQITK and $^{235}$WKGPAKLLWK) in the C-terminal domain of HIV-1 IN contributed to the karyophilic property of the protein. Consistent with us, Maertens *et al* also showed that the fusion protein of HIV-1 IN C-terminal fragment alone with GFP was localized to the nucleus, suggesting that the C-terminal domain may have a role in HIV-1 nuclear import (Maertens, Cherepanov et al. 2003). The mechanism underlying the nuclear localization function of IN C-terminus is still unclear. Since IN need have a NLS and/or bind to other haryophilic protein(s) for nuclear translocation, the two tri-lysine regions in the C-terminal domain of IN might be involved in the interaction with cellular nuclear import receptors(s). Thus, it is interesting to investigate the interaction between IN and cellular nuclear import receptor(s) such as Impα, Impβ, Imp7 and the contribution of the interaction to the nuclear import of HIV-1 PIC. On the other hand, it was suggested that the IN nuclear accumulation could be facilitated by its affinity for DNA (Devroe, Engelman et al. 2003). Because the C-terminal domain (213-288) is the least conserved of three domains of IN and binds DNA nonspecifically (Bushman, Engelman et al. 1993; Engelman, Bushman et al. 1993; van Gent, Vink et al.

1993), we could not exclude the possibility that the IN C-terminal substitution mutants may change their ability to bind DNA or to alter their DNA target ability, which would consequently affect IN nuclear accumulation. To obtain further information about the DNA binding ability of IN mutants, we can use biochemical subcellular fractionation experiment to check whether $IN_{KK215,9AA}$ or $IN_{KK240,4AE}$ still can enter the nucleus but without associating with chromatin.

We next evaluated the impact of nuclear localization-defective IN mutants on HIV-1 replication by using previously described HIV-1RT/IN trans-complement single-cycle replication system that comprised a RT/IN/Vpr/Env gene-deleted HIV-1 provirus, in which the *nef* gene was replaced by a firefly luciferase gene, and a Vpr-RT-IN expressor. There were three interesting findings from our infection data. First, all three IN C-terminal mutant viruses (KK215,9AA, KK240,4AE and RK263,4AA) could not replicate in Hela-CD4-CCR5-β-Gal cells, dividing and non-dividing C8166 T cells. Compared with D64E, the much lower levels of β-Gal positive cells and Luc activity of three IN C-terminal mutants suggest that they may affect early steps such as reverse transcription and/or nuclear import and consequently result in a reduced level of viral DNA in the nucleus. Second, by using semi-quantity PCR and Southern blot, We found that all of these IN mutants, especially KK240,4AA, negatively affected reverse transcription step. Previous studies have demonstrated that IN was required for reverse transcription in infected cells and have shown the C-terminal domain of IN was able to bind to heterodimeric RT in *in vitro* (Wu, Liu et al. 1999; Hehl, Joshi et al. 2004; Zhu, Dobard et al. 2004). It is possible that these C-terminal mutants, especially for KK240,4AE, may disrupt the interaction between IN and RT and result in decreased viral cDNA synthesis. Our results provide useful information for further studies aim at understanding the dynamics of RT-IN interactions during viral reverse transcription *in vivo*. Finally, Our results demonstrated that the two tri-lysine mutants, especially KK215,9AA, significantly impaired nuclear import step of HIV-1 replication. KK215,9AA,

KK240,4AE and RK263,4AA viruses are Class II IN mutant viruses since they affect multiple steps of the viral replication cycle. However, the truth that KK215,9AA significantly impaired viral DNA nuclear import suggests that the region $^{211}$KELQKQITK is in fact important for helping direct PICs to nucleus. The mechanism underlying the action of these IN mutants during HIV-1 DNA nuclear import still requires further investigation.

How does HIV-1 IN contribute to viral PIC nuclear import? It has been known that protein translocation from cytoplasm to nucleus can be mediated by different nuclear import pathway. In addition to the classic importinα (Impα) mediated nuclear transport pathway, importinβ (Impβ) was shown to bind to and import proteins independently of impα including HIV-1 Tat, Rev and HTLV Rex. Moreover, Imp7 and Imp8 have been found to contribute to the nuclear import of ribosomal proteins (Jakel and Gorlich 1998), glucocorticoid receptor (Freedman and Yamamoto 2004), histone H1 (Jakel, Albig et al. 1999; Bauerle, Doenecke et al. 2002), signal recognition particle protein 19 (Dean, von Ahsen et al. 2001). Therefore, we hypothesis that HIV-1 IN could interact with cellular nuclear import receptor(s) and contribute to the nuclear import of HIV-1 PIC.

By using a cell-based co-immunoprecipitation approach, we have investigated the potential interactions of HIV-1 IN with several cellular nuclear import factors. Our results have clearly shown that HIV-1 IN, in both IN-YFP and YFP-IN fusion protein forms, specifically interacted with Imp7, but not Imp8 and Impα. More interestingly, our deletion analysis indicated that the Imp7-binding sites lies in the C-terminal domain of IN. Strikingly, the substitute mutation analysis revealed that two regions $^{235}$WKGPAKLLWKG and $^{262}$RRKAK within the C-terminal domain of IN contribute to the IN/Imp7 interaction. Even though the infection analysis revealed that the vKKRK virus containing the Imp7-binding defect IN mutant is replication defective, the virus displayed impairments not only at nuclear import but also at viral reverse transcription. On the other hand, our

results showed that depletion of Imp7 in both HIV-1 producing and target cells lead to 2.5 to 3.5-fold decrease of HIV-1 infection, indicationg that Imp7 in fact contributes to an efficient HIV-1 replication. However, the mechanism underlying the inhibitory effect of Imp7 knockdown on viral replication still remains to be defined. Futhermore, this result a leads us to consider the possibility that HIV-1 IN may have the ability to interact with multiple cellular nuclear import factors, and dissociation of one of them could not abolish HIV-1 replication.

More recently, by using different experimental condition, we further demonstrated an interaction between HIV-1 IN and Impβ (including endogenous impβ). There are two interesting findings from our study: 1) The genetic analysis indicated that the region, which is responsible for IN interacting with Impβ, located again in the C-terminal domain of IN; 2) Immunoprecipitation of IN could simultaneously pull down both endogenous Impβ and Imp7. Therefore, the most plausible hypothesis at this time is that IN may recruit different cellular nuclear transport machineries, including Impβ and Imp7, to ensure an efficient HIV-1 nuclear import in both dividing and non-dividing cells. However, there are some remaining questions regarding the role of IN interaction with Imp7 or Impβ that need to be addressed in the future study.

First, how does the C-terminal domain of IN coordinate with two cellular importins and contribute to an efficient HIV-1 nuclear import? To address this question, we can perform more detailed mutagenic analysis to define the critical domain(s) and amino acid(s) in the C-terminal domain of IN for these viral/cellular protein interactions. Second, is there the possibility that IN interacts with a previously described Impβ/Imp7 heterodimer? The answer for this question has important implication for understand molecular basis of how different importins contribute to IN's action during HIV-1 replication. Fortunately, it was known that the last 30 aa of Imp7 was a critical binding site for Impβ and deletion of this region completely abolished its Impβ-binding ability (Bauerle, Doenecke

et al. 2002). So, construction of a T7-Imp7 with last 30aa deletion and co-IP experiment should give us more information. Third, whether Impα is required for Impβ-IN interaction? If co-IP system have detect that IN also binds to Impα in the same condition of the IN/Impβ interaction, the competition experiment by using SV40 T antigen NLS or Impβ-binding (IBB) domain of Impα will provide an opportunity for us to further exclude this possibility that IN directly binds to Impα. Finally and most importantly, how to validate the functional role of the interaction of IN with Imp7 and Impβ for HIV-1 nuclear import and replication? We can analyze the effect of Impβ- and Imp7-binding defective IN mutant(s) on viral nuclear import and replication. Another valuable approach is to directly target Impβ and Imp7 expression. Even though siRNA-mediated knockdown of Imp7 inhibits HIV-1 nuclear import and infection has been controversial (Fassati, Gorlich et al. 2003; Zielske and Stevenson 2005), it is possible that knock-down one such factor in the cell may not be sufficient to affect HIV-1 infection since our results revealed that HIV-1 IN interacts with two cellular nuclear import receptors.

Taken together, the currently available data suggest that HIV IN plays an important role in the nuclear import of the PIC, but requires the supports of the DNA flap, MA and Vpr proteins. It will be important to investigate how these factors coordinate with each other in order to maximize the capacity of HIV-1 to translocate into the nucleus of infected cells. We believe that all of those proposed studies will help us better understand the HIV-1 replication cycle and teach us how to stop the infection.

## REFERENCES


Adam, S. A. (1999). Transport pathways of macromolecules between the nucleus and the cytoplasm. *Curr Opin Cell Biol* 11(3), 402-6.

Adam, S. A., Adam, E. J., Chi, N. C., and Visser, G. D. (1995). Cytoplasmic factors in NLS-mediated targeting to the nuclear pore complex. *Cold Spring Harb Symp Quant Biol* 60, 687-94.

Aiken, C. (1997). Pseudotyping human immunodeficiency virus type 1 (HIV-1) by the glycoprotein of vesicular stomatitis virus targets HIV-1 entry to an endocytic pathway and suppresses both the requirement for Nef and the sensitivity to cyclosporin A. *J Virol* 71(8), 5871-7.

Aiken, C., and Trono, D. (1995). Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. *J Virol* 69(8), 5048-56.

Alroy, I., Tuvia, S., Greener, T., Gordon, D., Barr, H. M., Taglicht, D., Mandil-Levin, R., Ben-Avraham, D., Konforty, D., Nir, A., Levius, O., Bicoviski, V., Dori, M., Cohen, S., Yaar, L., Erez, O., Propheta-Meiran, O., Koskas, M., Caspi-Bachar, E., Alchanati, I., Sela-Brown, A., Moskowitz, H., Tessmer, U., Schubert, U., and Reiss, Y. (2005). The trans-Golgi network-associated human ubiquitin-protein ligase POSH is essential for HIV type 1 production. *Proc Natl Acad Sci U S A* 102(5), 1478-83.

Ansari-Lari, M. A., Donehower, L. A., and Gibbs, R. A. (1995). Analysis of human immunodeficiency virus type 1 integrase mutants. *Virology* 213(2), 680.

Ansari-Lari, M. A., and Gibbs, R. A. (1996). Expression of human immunodeficiency virus type 1 reverse transcriptase in trans during virion release and after infection. *J. Virol.* 70, 3870-3876.

Ao, Z., Fowke, K. R., Cohen, E. A., and Yao, X. (2005). Contribution of the C-terminal tri-lysine regions of human immunodeficiency virus type 1 integrase for efficient reverse transcription and viral DNA nuclear import. *Retrovirology* 2, 62.

Ao, Z., Yao, X., and Cohen, E. A. (2004). Assessment of the role of the central DNA flap in human immunodeficiency virus type 1 replication by using a single-cycle replication system. *J Virol* 78(6), 3170-7.

Appa, R. S., Shin, C. G., Lee, P., and Chow, S. A. (2001). Role of the nonspecific DNA-binding region and alpha helices within the core domain of retroviral integrase in selecting target DNA sites for integration. *J Biol Chem* 276(49), 45848-55.

Armon-Omer, A., Graessmann, A., and Loyter, A. (2004). A synthetic peptide bearing the HIV-1 integrase 161-173 amino acid residues mediates active nuclear import and binding to importin alpha: characterization of a functional nuclear localization signal. *J Mol Biol* 336(5), 1117-28.

**Baraz, L., Friedler, A., Blumenzweig, I., Nussinuv, O., Chen, N., Steinitz, M., Gilon, C., and Kotler, M. (1998). Human immunodeficiency virus type 1 Vif-derived peptides inhibit the viral protease and arrest virus production. *FEBS Lett* 441(3), 419-26.**

**Barreca, M. L., Lee, K. W., Chimirri, A., and Briggs, J. M. (2003). Molecular dynamics studies of the wild-type and double mutant HIV-1 integrase complexed with the 5CITEP inhibitor: mechanism for inhibition and drug resistance. *Biophys J* 84(3), 1450-63.**

**Bauerle, M., Doenecke, D., and Albig, W. (2002). The requirement of H1 histones for a heterodimeric nuclear import receptor. *J. Biol. Chem.* 277, 32480-9.**

**Bayliss, R., Littlewood, T., and Stewart, M. (2000). Structural basis for the interaction between FxFG nucleoporin repeats and importin-beta in nuclear trafficking. *Cell* 102(1), 99-108.**

**Ben-Efraim, I., and Gerace, L. (2001). Gradient of increasing affinity of importin beta for nucleoporins along the pathway of nuclear import. *J Cell Biol* 152(2), 411-7.**

**Berkowitz, R. D., Ohagen, A., Hoglund, S., and Goff, S. P. (1995). Retroviral nucleocapsid domains mediate the specific recognition of genomic viral RNAs by chimeric Gag polyproteins during RNA packaging in vivo. *J Virol* 69(10), 6445-56.**

**Bieniasz, P. D., Weiss, R. A., and McClure, M. O. (1995). Cell cycle dependence of foamy retrovirus infection. *J Virol* 69(11), 7295-9.**

**Bonnenfant, S., Thomas, C. M., Vita, C., Subra, F., Deprez, E., Zouhiri, F., Desmaele, D., D'Angelo, J., Mouscadet, J. F., and Leh, H. (2004). Styrylquinolines, integrase inhibitors acting prior to integration: a new mechanism of action for anti-integrase agents. *J Virol* 78(11), 5728-36.**

**Borman, A. M., Quillent, C., Charneau, P., Dauguet, C., and Clavel, F. (1995). Human immunodeficiency virus type 1 Vif- mutant particles from restrictive cells: role of Vif in correct particle assembly and infectivity. *J Virol* 69(4), 2058-67.**

**Bouyac-Bertoia, M., Dvorin, J. D., Fouchier, R. A., Jenkins, Y., Meyer, B. E., Wu, L. I., Emerman, M., and Malim, M. H. (2001). HIV-1 infection requires a functional integrase NLS. *Mol Cell* 7(5), 1025-35.**

**Braaten, D., Aberham, C., Franke, E. K., Yin, L., Phares, W., and Luban, J. (1996). Cyclosporine A-resistant human immunodeficiency virus type 1 mutants demonstrate that Gag encodes the functional target of cyclophilin A. *J Virol* 70(8), 5170-6.**

**Brussel, A., Mathez, D., Broche-Pierre, S., Lancar, R., Calvez, T., Sonigo, P., and Leibowitch, J. (2003). Longitudinal monitoring of 2-long terminal repeat circles in peripheral blood mononuclear cells from patients with chronic HIV-1 infection. *Aids* 17(5), 645-52.**

**Brussel, A., and Sonigo, P. (2004). Evidence for gene expression by unintegrated human immunodeficiency virus type 1 DNA species. *J Virol* 78(20), 11263-71.**

**Bukovsky, A., and Gottlinger, H. (1996). Lack of integrase can markedly affect human immunodeficiency virus type 1 particle production in the presence of an active viral protease. *J Virol* 70(10), 6820-5.**

**Bukovsky, A. A., Song, J. P., and Naldini, L. (1999). Interaction of human immunodeficiency virus-derived vectors with wild-type virus in transduced cells. *J Virol* 73(8), 7087-92.**

**Bukrinsky, M., and Haffar, O. K. (1998a). HIV-1 nuclear import: matrx protein is back on center stage, this time togather with Vpr. *Molecular Medicine* 4, 138-143.**

**Bukrinsky, M., Haggerty, S., Dempsey, M. P., I.N., S., Adzhubei, A., Spitz, L., Lewis, P., Goldfarb, D., Emerman, M., and Stevenson, M. (1993). A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. *Nature* 365, 666-669.**

**Bukrinsky, M., I.N., S., Dempsey, M. P., Stanwick, T. L., Bukrinskaya, A. G., Haggerty, S., and Stevenson, M. (1992). Active nuclear import of human immunodeficiency virus type 1 preintegration complex. *Proc Natl Acad Sci U S A* 89, 6580-6584.**

**Bukrinsky, M. I., and Haffar, O. K. (1998b). HIV-1 nuclear import: matrix protein is back on center stage, this time together with Vpr. *Mol Med* 4(3), 138-43.**

**Bukrinsky, M. I., Sharova, N., McDonald, T. L., Pushkarskaya, T., Tarpley, W. G., and Stevenson, M. (1993). Association of integrase, matrix, and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. *Proc Natl Acad Sci U S A* 90(13), 6125-9.**

**Bushman, F. (2002). Targeting retroviral integration? *Mol Ther* 6(5), 570-1.**

**Bushman, F. (2003). Measuring covert HIV replication during HAART: the abundance of 2-LTR circles is not a reliable marker. *Aids* 17(5), 749-50.**

**Bushman, F. D., Engelman, A., Palmer, I., Wingfield, P., and Craigie, R. (1993). Domains of the integrase protein of human immunodeficiency virus type 1 responsible for polynucleotidyl transfer and zinc binding. *Proc Natl Acad Sci U S A* 90(8), 3428-32.**

**Busschots, K., Vercammen, J., Emiliani, S., Benarous, R., Engelborghs, Y., Christ, F., and Debyser, Z. (2005). The interaction of LEDGF/p75 with integrase is lentivirus-specific and promotes DNA binding. *J Biol Chem* 280(18), 17841-7.**

**Butler, S. L., Johnson, E. P., and Bushman, F. D. (2002). Human immunodeficiency virus cDNA metabolism: notable stability of two-long terminal repeat circles. *J Virol* 76(8), 3739-47.**

**Cai, M., Huang, Y., Zheng, R., Wei, S. Q., Ghirlando, R., Lee, M. S., Craigie, R., Gronenborn, A. M., and Clore, G. M. (1998). Solution structure of the cellular factor BAF responsible for protecting retroviral DNA from autointegration. *Nat Struct Biol* 5(10), 903-9.**

**Cannon, P. M., Byles, E. D., Kingsman, S. M., and Kingsman, A. J. (1996). Conserved sequences in the carboxyl terminus of integrase that are essential for human immunodeficiency virus type 1 replication. *J Virol* 70(1), 651-7.**

**Cereseto, A., Manganaro, L., Gutierrez, M. I., Terreni, M., Fittipaldi, A., Lusic, M., Marcello, A., and Giacca, M. (2005). Acetylation of HIV-1 integrase by p300 regulates viral integration. *Embo J* 24(17), 3070-81.**

**Charneau, P., Alizon, M., and Clavel, F. (1992). A second origin of plus strand sythesis is required for optimal HIV replication. *J Virol* 66, 2814-2820.**

**Charneau, P., Mirambeau, G., Roux, P., Paulous, S., Buc, H., and Clavel, F. (1994). HIV-1 reverse transcription. A termination step at the center of the genome. *J Mol Biol* 241(5), 651-62.**

**Chen, H., and Engelman, A. (1998). The barrier-to-autointegration protein is a host factor for HIV type 1 integration. *Proc Natl Acad Sci U S A* 95(26), 15270-4.**

**Chen, H., Wei, S. Q., and Engelman, A. (1999). Multiple integrase functions are required to form the native structure of the human immunodeficiency virus type I intasome. *J Biol Chem* 274(24), 17358-64.**

**Chen, J. C., Krucinski, J., Miercke, L. J., Finer-Moore, J. S., Tang, A. H., Leavitt, A. D., and Stroud, R. M. (2000). Crystal structure of the HIV-1 integrase catalytic core and C-terminal domains: a model for viral DNA binding. *Proc Natl Acad Sci U S A* 97(15), 8233-8.**

**Cherepanov, P., Ambrosio, A. L., Rahman, S., Ellenberger, T., and Engelman, A. (2005). Structural basis for the recognition between HIV-1 integrase and transcriptional coactivator p75. *Proc Natl Acad Sci U S A* 102(48), 17308-13.**

**Cherepanov, P., Devroe, E., Silver, P. A., and Engelman, A. (2004). Identification of an evolutionarily conserved domain in human lens epithelium-derived growth factor/transcriptional co-activator p75 (LEDGF/p75) that binds HIV-1 integrase. *J Biol Chem* 279(47), 48883-92.**

**Cherepanov, P., Maertens, G., Proost, P., Devreese, B., Beeumen, J. V., Engelborghs, Y., De Clercq, E. D., and Debyser, Z. (2003). HIV-1**

integrase forms stable tetramers and associates with LEDGF/p75 protein in human cells. *J. Biol. Chem.* 278, 372-381.

Chow, S. A., Vincent, K. A., Ellison, V., and Brown, P. O. (1992). Reversal of integration and DNA splicing mediated by integrase of human immunodeficiency virus. *Science* 255(5045), 723-6.

Chun, T. W., Carruth, L., Finzi, D., Shen, X., DiGiuseppe, J. A., Taylor, H., Hermankova, M., Chadwick, K., Margolick, J., Quinn, T. C., Kuo, Y. H., Brookmeyer, R., Zeiger, M. A., Barditch-Crovo, P., and Siliciano, R. F. (1997). Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature* 387(6629), 183-8.

Chun, T. W., Stuyver, L., Mizell, S. B., Ehler, L. A., Mican, J. A., Baseler, M., Lloyd, A. L., Nowak, M. A., and Fauci, A. S. (1997). Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proc Natl Acad Sci U S A* 94(24), 13193-7.

Cingolani, G., Bednenko, J., Gillespie, M. T., and Gerace, L. (2002). Molecular basis for the recognition of a nonclassical nuclear localization signal by importin beta. *Mol Cell* 10(6), 1345-53.

Cingolani, G., Petosa, C., Weis, K., and Muller, C. W. (1999). Structure of importin-beta bound to the IBB domain of importin-alpha. *Nature* 399(6733), 221-9.

Ciuffi, A., Llano, M., Poeschla, E., Hoffmann, C., Leipzig, J., Shinn, P., Ecker, J. R., and Bushman, F. (2005). A role for LEDGF/p75 in targeting HIV DNA integration. *Nat Med* 11(12), 1287-9.

Coffin, J. M. (1990). Retroviridae and their replication. *In Fields.B.N and Knipe.D.M. (eds). Fields Virology 2nd. Raven Press. N.Y.* 2, 1437-1500.

Cohen, E. A., Dehni, G., Sodroski, J. G., and Haseltine, W. A. (1990). Human immunodeficiency virus vpr product is a virion-associated regulatory protein. *J Virol* 64(6), 3097-9.

Cohen, E. A., Terwilliger, E. F., Sodroski, J. G., and Haseltine, W. A. (1988). Identification of a protein encoded by the vpu gene of HIV-1. *Nature* 334(6182), 532-4.

Connor, R. I., Chen, B. K., Choe, S., and Landau, N. R. (1995). Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. *Virology* 206(2), 935-44.

Cronshaw, J. M., Krutchinsky, A. N., Zhang, W., Chait, B. T., and Matunis, M. J. (2002). Proteomic analysis of the mammalian nuclear pore complex. *J Cell Biol* 158(5), 915-27.

Cuomo, C., Kirch, S., Gyuris, J., Brent, R., and Oettinger, M. (1994). Rch1, a protein that specifically interact withs with the RAG-1 recombination-activating protein. *Proc. Natl. Acad. Sci. USA* 91, 6156-6160.

Damgaard, C. K., Dyhr-Mikkelsen, H., and Kjems, J. (1998). Mapping the RNA binding sites for human immunodeficiency virus type-1 gag and

NC proteins within the complete HIV-1 and -2 untranslated leader regions. *Nucleic Acids Res* 26(16), 3667-76.

d'Angelo, J., Mouscadet, J. F., Desmaele, D., Zouhiri, F., and Leh, H. (2001). HIV-1 integrase: the next target for AIDS therapy? *Pathol Biol (Paris)* 49(3), 237-46.

Daniel, R., Katz, R. A., and Skalka, A. M. (1999). A role for DNA-PK in retroviral DNA integration. *Science* 284(5414), 644-7.

Dardalhon, V., Herpers, B., Noraz, N., Pflumio, F., Guetard, D., Leveau, C., Dubart-Kupperschmitt, A., Charneau, P., and Taylor, N. (2001). Lentivirus-mediated gene transfer in primary T cells is enhanced by a central DNA flap. *Gene Ther* 8(3), 190-8.

Dasgupta, A., and Wilson, D. W. (1999). ATP depletion blocks herpes simplex virus DNA packaging and capsid maturation. *J Virol* 73(3), 2006-15.

De Guzman, R. N., Wu, Z. R., Stalling, C. C., Pappalardo, L., Borer, P. N., and Summers, M. F. (1998). Structure of the HIV-1 nucleocapsid protein bound to the SL3 psi-RNA recognition element. *Science* 279(5349), 384-8.

De Luca, L., Vistoli, G., Pedretti, A., Barreca, M. L., and Chimirri, A. (2005). Molecular dynamics studies of the full-length integrase-DNA complex. *Biochem Biophys Res Commun* 336(4), 1010-6.

de Noronha, C. M., Sherman, M. P., Lin, H. W., Cavrois, M. V., Moir, R. D., Goldman, R. D., and Greene, W. C. (2001). Dynamic disruptions in nuclear envelope architecture and integrity induced by HIV-1 Vpr. *Science* 294(5544), 1105-8.

De Rijck, J., Van Maele, B., and Debyser, Z. (2005). Positional effects of the central DNA flap in HIV-1-derived lentiviral vectors. *Biochem Biophys Res Commun* 328(4), 987-94.

Dean, K. A., von Ahsen, O., Gorlich, D., and Fried, H. M. (2001). Signal recognition particle protein 19 is imported into the nucleus by importin 8 (RanBP8) and transportin. *J Cell Sci* 114(Pt 19), 3479-85.

Dejesus E, B. D., Markowitz M, et al. (2006). The HIV integrase inhibitor GS-9137 (JTK-303) exhibits potent antiviral activity in treatment-naive and experienced patients (abstract LB160). *XIII CROI. Denver CO, February 5-8.*

Depienne, C., Mousnier, A., Leh, H., Rouzic, E. L., Dormont, D., Benichou, S., and Dargemont, C. (2001). Characterization of the nuclear import pathway for HIV-1 integrase. *J Biol Chem* 276, 18192-18107.

Depienne, C., Roques, P., Creminon, C., Fritsch, L., Casseron, R., Dormont, D., Dargemont, C., and Benichou, S. (2000). Cellular distribution and karyophilic properties of matrix, integrase, and Vpr proteins from the human and simian immunodeficiency viruses. *Exp Cell Res* 260(2), 387-95.

**Derdowski, A., Ding, L., and Spearman, P. (2004). A novel fluorescence resonance energy transfer assay demonstrates that the human immunodeficiency virus type 1 Pr55Gag I domain mediates Gag-Gag interactions. *J Virol* 78(3), 1230-42.**

**Dettenhofer, M., Cen, S., Carlson, B. A., Kleiman, L., and Yu, X. F. (2000). Association of human immunodeficiency virus type 1 Vif with RNA and its role in reverse transcription. *J Virol* 74(19), 8938-45.**

**Devroe, E., Engelman, A., and Silver, P. A. (2003). Intracellular transport of human immunodeficiency virus type 1 integrase. *J Cell Sci* 116(Pt 21), 4401-8.**

**Di Marzio, P., Choe, S., Ebright, M., Knoblauch, R., and Landau, N. R. (1995). Mutational analysis of cell cycle arrest, nuclear localization and virion packaging of human immunodeficiency virus type 1 Vpr. *J Virol* 69(12), 7909-16.**

**Dirac, A. M., and Kjems, J. (2001). Mapping DNA-binding sites of HIV-1 integrase by protein footprinting. *Eur J Biochem* 268(3), 743-51.**

**Dismuke, D and Aiken, C. (2006).Evidence for a functional link between uncoating of the human immunodeficiency virus type 1 core and nuclear import of the viral preintegration complex. *J Virol.* 80(8), 3712-20.**

**Dupont, S., Sharova, N., DeHoratius, C., Virbasius, C. M., Zhu, X., Bukrinskaya, A. G., Stevenson, M., and Green, M. R. (1999). A novel nuclear export activity in HIV-1 matrix protein required for viral replication. *Nature* 402(6762), 681-5.**

**Dvorin, J. D., Bell, P., Maul, G. G., Yamashita, M., Emerman, M., and Malim, M. H. (2002). Reassessment of the roles of integrase and the central DNA flap in human immunodeficiency virus type 1 nuclear import. *J Virol* 76(23), 12087-96.**

**Dvorin, J. D., and Malim, M. H. (2003). Intracellular trafficking of HIV-1 cores: journey to the center of the cell. *Curr Top Microbiol Immunol* 281, 179-208.**

**Dyda, F., Hickman, A. B., Jenkins, T. M., Engelman, A., Craigie, R., and Davies, D. R. (1994). Crystal structure of the catalytic domain of HIV-1 integrase: similarity to other polynucleotidyl transferases. *Science* 266(5193), 1981-6.**

**Ebina, H., Aoki, J., Hatta, S., Yoshida, T., and Koyanagi, Y. (2004). Role of Nup98 in nuclear entry of human immunodeficiency virus type 1 cDNA. *Microbes Infect* 6(8), 715-24.**

**Eckstein, D. A., Sherman, M. P., Penn, M. L., Chin, P. S., De Noronha, C. M., Greene, W. C., and Goldsmith, M. A. (2001). HIV-1 Vpr enhances viral burden by facilitating infection of tissue macrophages but not nondividing CD4+ T cells. *J Exp Med* 194(10), 1407-19.**

**Eijkelenboom, A. P., Lutzke, R. A., Boelens, R., Plasterk, R. H., Kaptein, R., and Hard, K. (1995). The DNA-binding domain of HIV-1 integrase has an SH3-like fold.** ***Nat Struct Biol*** **2(9), 807-10.**

**Eijkelenboom, A. P., Sprangers, R., Hard, K., Puras Lutzke, R. A., Plasterk, R. H., Boelens, R., and Kaptein, R. (1999). Refined solution structure of the C-terminal DNA-binding domain of human immunovirus-1 integrase.** ***Proteins*** **36(4), 556-64.**

**Ellison, V., and Brown, P. O. (1994). A stable complex between integrase and viral DNA ends mediates human immunodeficiency virus integration in vitro.** ***Proc Natl Acad Sci U S A*** **91(15), 7316-20.**

**Ellison, V., Gerton, J., Vincent, K. A., and Brown, P. O. (1995). An essential interaction between distinct domains of HIV-1 integrase mediates assembly of the active multimer.** ***J Biol Chem*** **270(7), 3320-6.**

**Emiliani, S., Mousnier, A., Busschots, K., Maroun, M., Van Maele, B., Tempe, D., Vandekerckhove, L., Moisant, F., Ben-Slama, L., Witvrouw, M., Christ, F., Rain, J. C., Dargemont, C., Debyser, Z., and Benarous, R. (2005). Integrase mutants defective for interaction with LEDGF/p75 are impaired in chromosome tethering and HIV-1 replication.** ***J Biol Chem*** **280(27), 25517-23.**

**Engelman, A. (1999). In vivo analysis of retroviral integrase structure and function.** ***Adv Virus Res*** **52, 411-26.**

**Engelman, A., Bushman, F. D., and Craigie, R. (1993). Identification of discrete functional domains of HIV-1 integrase and their organization within an active multimeric complex.** ***Embo J*** **12(8), 3269-75.**

**Engelman, A., and Craigie, R. (1992). Identification of conserved amino acid residues critical for human immunodeficiency virus type 1 integrase function in vitro.** ***J Virol*** **66(11), 6361-9.**

**Engelman, A., and Craigie, R. (1995). Efficient magnesium-dependent human immunodeficiency virus type 1 integrase activity.** ***J Virol*** **69(9), 5908-11.**

**Engelman, A., Englund, G., Orenstein, J. M., Martin, M. A., and Craigie, R. (1995). Multiple effects of mutations in human immunodeficiency virus type 1 integrase on viral replication.** ***J Virol*** **69(5), 2729-36.**

**Engelman, A., Hickman, A. B., and Craigie, R. (1994). The core and carboxyl-terminal domains of the integrase protein of human immunodeficiency virus type 1 each contribute to nonspecific DNA binding.** ***J Virol*** **68(9), 5911-7.**

**Engelman, A., Liu, Y., Chen, H., Farzan, M., and Dyda, F. (1997). Structure-based mutagenesis of the catalytic domain of human immunodeficiency virus type 1 integrase.** ***J Virol*** **71(5), 3507-14.**

**Esposito, D., and Craigie, R. (1998). Sequence specificity of viral end DNA binding by HIV-1 integrase reveals critical regions for protein-DNA interaction.** ***Embo J*** **17(19), 5832-43.**

**Farnet, C. M., and Bushman, F. D. (1996). HIV cDNA integration: molecular biology and inhibitor development. *Aids* 10 Suppl A, S3-11.**

**Farnet, C. M., and Bushman, F. D. (1997). HIV-1 cDNA integration: requirement of HMG I(Y) protein for function of preintegration complexes in vitro. *Cell* 88(4), 483-92.**

**Farnet, C. M., and Haseltine, W. A. (1991). Circularization of human immunodeficiency virus type 1 DNA in vitro. *J Virol* 65(12), 6942-52.**

**Fassati, A., Gorlich, D., Harrison, I., Zaytseva, L., and Mingot, J. M. (2003). Nuclear import of HIV-1 intracellular reverse transcription complexes is mediated by importin 7. *Embo J* 22(14), 3675-85.**

**Fikkert, V., Van Maele, B., Vercammen, J., Hantson, A., Van Remoortel, B., Michiels, M., Gurnari, C., Pannecouque, C., De Maeyer, M., Engelborghs, Y., De Clercq, E., Debyser, Z., and Witvrouw, M. (2003). Development of resistance against diketo derivatives of human immunodeficiency virus type 1 by progressive accumulation of integrase mutations. *J Virol* 77(21), 11459-70.**

**Fisher, A. G., Feinberg, M. B., Josephs, S. F., Harper, M. E., Marselle, L. M., Reyes, G., Gonda, M. A., Aldovini, A., Debouk, C., Gallo, R. C., and et al. (1986). The trans-activator gene of HTLV-III is essential for virus replication. *Nature* 320(6060), 367-71.**

**Fletcher, T. M., 3rd, Soares, M. A., McPhearson, S., Hui, H., Wiskerchen, M., Muesing, M. A., Shaw, G. M., Leavitt, A. D., Boeke, J. D., and Hahn, B. H. (1997). Complementation of integrase function in HIV-1 virions. *Embo J* 16(16), 5123-38.**

**Follenzi, A., Ailles, L. E., Bakovic, S., Geuna, M., and Naldini, L. (2000). Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat Genet* 25(2), 217-22.**

**Forget, J., Yao, X. J., Mercier, J., and Cohen, E. A. (1998). Human immunodeficiency virus type 1 vpr protein transactivation function: mechanism and identification of domains involved. *J Mol Biol* 284(4), 915-23.**

**Fouchier, R. A., and Malim, M. H. (1999). Nuclear import of human immunodeficiency virus type-1 preintegration complexes. *Adv Virus Res* 52, 275-99.**

**Fouchier, R. A., Meyer, B. E., Simon, J. H., Fischer, U., Albright, A. V., Gonzalez-Scarano, F., and Malim, M. H. (1998). Interaction of the human immunodeficiency virus type 1 Vpr protein with the nuclear pore complex. *J Virol* 72(7), 6004-13.**

**Fouchier, R. A., Meyer, B. E., Simon, J. H., Fisher, U., and Malim, M. H. (1997). HIV-1 infection of non-dividing cells: evidence that the amino-terminal basic region of the viral matrix protein is important for Gag processing but not for post-entry nuclear import. *Embo J* 16, 4531-4539.**

**Franke, E. K., Yuan, H. E., and Luban, J. (1994). Specific incorporation of cyclophilin A into HIV-1 virions. *Nature* 372(6504), 359-62.**

**Freed, E. O. (1998). HIV-1 gag proteins: diverse functions in the virus life cycle. *Virology* 251(1), 1-15.**

**Freed, E. O., Englund, G., and Martin, M. A. (1995). Role of the basic domain of human immunodeficiency virus type 1 matrix in macrophage infection. *J Virol* 69, 3949-3954.**

**Freedman, N. D., and Yamamoto, K. R. (2004). Importin 7 and importin alpha/importin beta are nuclear import receptors for the glucocorticoid receptor. *Mol Biol Cell* 15(5), 2276-86.**

**Fridell, R. A., Truant, R., Thorne, L., Benson, R. E., and Cullen, B. R. (1997). Nuclear import of hnRNP A1 is mediated by a novel cellular cofactor related to karyopherin-beta. *J Cell Sci* 110 ( Pt 11), 1325-31.**

**Fried, H., and Kutay, U. (2003). Nucleocytoplasmic transport: taking an inventory. *Cell Mol Life Sci* 60(8), 1659-88.**

**Friedler, A., Blumenzweig, I., Baraz, L., Steinitz, M., Kotler, M., and Gilon, C. (1999). Peptides derived from HIV-1 Vif: a non-substrate based novel type of HIV-1 protease inhibitors. *J Mol Biol* 287(1), 93-101.**

**Furukawa, K. (1999). LAP2 binding protein 1 (L2BP1/BAF) is a candidate mediator of LAP2-chromatin interaction. *J Cell Sci* 112 ( Pt 15), 2485-92.**

**Gallay, P., Hope, T., Chin, D., and Trono, D. (1997). HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. *Proc Natl Acad Sci U S A* 94(18), 9825-30.**

**Gallay, P., Stitt, V., Mundy, C., Oettinger, M., and Trono, D. (1996). Role of the karyopherin pathway in human immunodeficiency virus type 1 nuclear import. *J Virol* 70(2), 1027-32.**

**Gallay, P., Swingler, S., Aiken, C., and Trono, D. (1995a). HIV-1 infection of nondividing cells: C-terminal tyrosine phosphorylation of the viral matrix protein is a key regulator. *cell* 80, 379-388.**

**Gallay, P., Swingler, S., Song, J., Bushman, F., and Trono, D. (1995b). HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. *Cell* 83(4), 569-76.**

**Gao, K., Butler, S. L., and Bushman, F. (2001). Human immunodeficiency virus type 1 integrase: arrangement of protein domains in active cDNA complexes. *Embo J* 20(13), 3565-76.**

**Garrus, J. E., von Schwedler, U. K., Pornillos, O. W., Morham, S. G., Zavitz, K. H., Wang, H. E., Wettstein, D. A., Stray, K. M., Cote, M., Rich, R. L., Myszka, D. G., and Sundquist, W. I. (2001). Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell* 107(1), 55-65.**

**Gartner, S., and Popovic, M. (1990). Macrophage tropism of HIV-1. *AIDS Res Hum Retroviruses* 8, 1017-1021.**

**Ge, H., Si, Y., and Roeder, R. G. (1998). Isolation of cDNAs encoding novel transcription coactivators p52 and p75 reveals an alternate regulatory mechanism of transcriptional activation. *Embo J* 17(22), 6723-9.**

**Gerton, J. L., Ohgi, S., Olsen, M., DeRisi, J., and Brown, P. O. (1998). Effects of mutations in residues near the active site of human immunodeficiency virus type 1 integrase on specific enzyme-substrate interactions. *J Virol* 72(6), 5046-55.**

**Golf, S. P. (1992). Genetics and Retroviral integration. *Annu. Rev. Genet.*(26), 527-544.**

**Goncalves, J., Korin, Y., Zack, J., and Gabuzda, D. (1996). Role of Vif in human immunodeficiency virus type 1 reverse transcription. *J Virol* 70(12), 8701-9.**

**Gorlich, D., Dabrowski, M., Bischoff, F. R., Kutay, U., Bork, P., Hartmann, E., Prehn, S., and Izaurralde, E. (1997). A novel class of RanGTP binding proteins. *J Cell Biol* 138(1), 65-80.**

**Gorlich, D., Kraft, R., Kostka, S., Vogel, F., Hartmann, E., Laskey, R. A., Mattaj, I. W., and Izaurraide, E. (1996). Importin provides a link between nuclear protein import and U snRNA export. *Cell* 87(1), 21-32.**

**Gorlich, D., and Kutay, U. (1999). Transport between the cell nucleus and the cytoplasm. *Annu Rev Cell Dev Biol* 15, 607-60.**

**Gorlich, D., and Mattaj, I. W. (1996). Nucleocytoplasmic transport. *Science* 271(5255), 1513-8.**

**Gottlinger, H. G., Dorfman, T., Sodroski, J. G., and Haseltine, W. A. (1991). Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. *Proc Natl Acad Sci U S A* 88(8), 3195-9.**

**Gould, S. J., Booth, A. M., and Hildreth, J. E. (2003). The Trojan exosome hypothesis. *Proc Natl Acad Sci U S A* 100(19), 10592-7.**

**Greene, W. C., and Peterlin, B. M. (2002). Charting HIV's remarkable voyage through the cell: Basic science as a passport to future therapy. *Nat Med* 8(7), 673-80.**

**Grinsztejn B, N. B., Katlama C, et al. (2006). Potent antiretroviral effect of MK-0518, a novel HIV-1 integrase inhibitor, in patients with triple-class resistant virus (abstract LB159). *XIII CROI. Denver CO, February 5-8.***

**Haffar, O. K., Popov, S., Dubrovsky, L., Agostini, I., Tang, H., Pushkarsky, T., Nadler, S. G., and Bukrinsky, M. (2000). Two nuclear localization signals in the HIV-1 matrix protein regulate nuclear import of the HIV-1 pre-integration complex. *J Mol Biol* 299(2), 359-68.**

**Harel, A., and Forbes, D. J. (2004). Importin beta: conducting a much larger cellular symphony. *Mol Cell* 16(3), 319-30.**

**Harel, A., Orjalo, A. V., Vincent, T., Lachish-Zalait, A., Vasu, S., Shah, S., Zimmerman, E., Elbaum, M., and Forbes, D. J. (2003). Removal of a single pore subcomplex results in vertebrate nuclei devoid of nuclear pores. *Mol Cell* 11(4), 853-64.**

**Harper, A. L., Skinner, L. M., Sudol, M., and Katzman, M. (2001). Use of patient-derived human immunodeficiency virus type 1 integrases to identify a protein residue that affects target site selection. *J Virol* 75(16), 7756-62.**

**Harrich, D., and Hooker, B. (2002). Mechanistic aspects of HIV-1 reverse transcription initiation. *Rev Med Virol* 12(1), 31-45.**

**Harrich, D., Ulich, C., Garcia-Martinez, L. F., and Gaynor, R. B. (1997). Tat is required for efficient HIV-1 reverse transcription. *Embo J* 16(6), 1224-35.**

**Harrich, D., Ulich, C., and Gaynor, R. B. (1996). A critical role for the TAR element in promoting efficient human immunodeficiency virus type 1 reverse transcription. *J Virol* 70(6), 4017-27.**

**Hatziioannou, T., and Goff, S. P. (2001). Infection of nondividing cells by Rous sarcoma virus. *J Virol* 75(19), 9526-31.**

**Hazuda, D., Blau, C. U., Felock, P., Hastings, J., Pramanik, B., Wolfe, A., Bushman, F., Farnet, C., Goetz, M., Williams, M., Silverman, K., Lingham, R., and Singh, S. (1999). Isolation and characterization of novel human immunodeficiency virus integrase inhibitors from fungal metabolites. *Antivir Chem Chemother* 10(2), 63-70.**

**Hazuda, D. J., Anthony, N. J., Gomez, R. P., Jolly, S. M., Wai, J. S., Zhuang, L., Fisher, T. E., Embrey, M., Guare, J. P., Jr., Egbertson, M. S., Vacca, J. P., Huff, J. R., Felock, P. J., Witmer, M. V., Stillmock, K. A., Danovich, R., Grobler, J., Miller, M. D., Espeseth, A. S., Jin, L., Chen, I. W., Lin, J. H., Kassahun, K., Ellis, J. D., Wong, B. K., Xu, W., Pearson, P. G., Schleif, W. A., Cortese, R., Emini, E., Summa, V., Holloway, M. K., and Young, S. D. (2004). A naphthyridine carboxamide provides evidence for discordant resistance between mechanistically identical inhibitors of HIV-1 integrase. *Proc Natl Acad Sci U S A* 101(31), 11233-8.**

**Hazuda, D. J., Felock, P., Witmer, M., Wolfe, A., Stillmock, K., Grobler, J. A., Espeseth, A., Gabryelski, L., Schleif, W., Blau, C., and Miller, M. D. (2000). Inhibitors of strand transfer that prevent integration and inhibit HIV-1 replication in cells. *Science* 287(5453), 646-50.**

**Hehl, E. A., Joshi, P., Kalpana, G. V., and Prasad, V. R. (2004). Interaction between human immunodeficiency virus type 1 reverse transcriptase and integrase proteins. *J Virol* 78(10), 5056-67.**

**Heinzinger, N. K., Bukinsky, M. I., Haggerty, S. A., Ragland, A. M., Kewalramani, V., Lee, M. A., Gendelman, H. E., Ratner, L., Stevenson, M., and Emerman, M. (1994). The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proc Natl Acad Sci U S A* 91, 7311-7315.**

**Herold, A., Truant, R., Wiegand, H., and Cullen, B. R. (1998). Determination of the functional domain organization of the importin alpha nuclear import factor. *J Cell Biol* 143(2), 309-18.**

**Heuer, T. S., and Brown, P. O. (1997). Mapping features of HIV-1 integrase near selected sites on viral and target DNA molecules in an active enzyme-DNA complex by photo-cross-linking. *Biochemistry* 36(35), 10655-65.**

**Heuer, T. S., and Brown, P. O. (1998). Photo-cross-linking studies suggest a model for the architecture of an active human immunodeficiency virus type 1 integrase-DNA complex. *Biochemistry* 37(19), 6667-78.**

**Ho, W. Z., Cherukuri, R., and Douglas, S. D. (1994). The macrophage and HIV-1. *Immunol Ser* 60, 569-587.**

**Hu, S. C., Court, D. L., Zweig, M., and Levin, J. G. (1986). Murine leukemia virus pol gene products: analysis with antisera generated against reverse transcriptase and endonuclease fusion proteins expressed in Escherichia coli. *J Virol* 60(1), 267-74.**

**Huang, M., Orenstein, J. M., Martin, M. A., and Freed, E. O. (1995). p6Gag is required for particle production from full-length human immunodeficiency virus type 1 molecular clones expressing protease. *J Virol* 69(11), 6810-8.**

**Ikeda, T., Nishitsuji, H., Zhou, X., Nara, N., Ohashi, T., Kannagi, M., and Masuda, T. (2004). Evaluation of the functional involvement of human immunodeficiency virus type 1 integrase in nuclear import of viral cDNA during acute infection. *J Virol* 78(21), 11563-73.**

**Innocenti, P., Ottmann, M., Morand, P., Leclercq, P., and J.M., S. (1992). HIV-1 in blood monocytes: frequency of detection of proviral DNA using PCR and comparison with the total CD4 count. *AIDS Res Hum Retroviruses* 8, 261-268.**

**Ishikawa, T., Okui, N., Kobayashi, N., Sakuma, R., Kitamura, T., and Kitamura, Y. (1999). Monoclonal antibodies against the minimal DNA-binding domain in the carboxyl-terminal region of human immunodeficiency virus type 1 integrase. *J Virol* 73(5), 4475-80.**

**Jakel, S., Albig, W., Kutay, U., Bischoff, F. R., Schwamborn, K., Doenecke, D., and Gorlich, D. (1999). The importin beta/importin 7 heterodimer is a functional nuclear import receptor for histone H1. *Embo J* 18(9), 2411-23.**

**Jakel, S., and Gorlich, D. (1998). Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. *Embo J* 17(15), 4491-502.**

**Jakel, S., Mingot, J. M., Schwarzmaier, P., Hartmann, E., and Gorlich, D. (2002). Importins fulfil a dual function as nuclear import receptors and cytoplasmic chaperones for exposed basic domains. *Embo J* 21(3), 377-86.**

**Jenkins, T. M., Engelman, A., Ghirlando, R., and Craigie, R. (1996). A soluble active mutant of HIV-1 integrase: involvement of both the core and carboxyl-terminal domains in multimerization. *J Biol Chem* 271(13), 7712-8.**

**Jenkins, T. M., Esposito, D., Engelman, A., and Craigie, R. (1997). Critical contacts between HIV-1 integrase and viral DNA identified by structure-based analysis and photo-crosslinking. *Embo J* 16(22), 6849-59.**

**Jenkins, T. M., Hickman, A. B., Dyda, F., Ghirlando, R., Davies, D. R., and Craigie, R. (1995). Catalytic domain of human immunodeficiency virus type 1 integrase: identification of a soluble mutant by systematic replacement of hydrophobic residues. *Proc Natl Acad Sci U S A* 92(13), 6057-61.**

**Jenkins, Y., McEntee, M., Weis, K., and Greene, W. C. (1998). Characterization of HIV-1 vpr nuclear import: analysis of signals and pathways. *J Cell Biol* 143(4), 875-85.**

**Johnson, A. A., Marchand, C., and Pommier, Y. (2004). HIV-1 integrase inhibitors: a decade of research and two drugs in clinical trial. *Curr Top Med Chem* 4(10), 1059-77.**

**Jordan, A., Defechereux, P., and Verdin, E. (2001). The site of HIV-1 integration in the human genome determines basal transcriptional activity and response to Tat transactivation. *Embo J* 20(7), 1726-38.**

**Julias, J. G., Ferris, A. L., Boyer, P. L., and Hughes, S. H. (2001). Replication of phenotypically mixed human immunodeficiency virus type 1 virions containing catalytically active and catalytically inactive reverse transcriptase. *J Virol* 75(14), 6537-46.**

**Kalpana, G. V., Marmon, S., Wang, W., Crabtree, G. R., and Goff, S. P. (1994). Binding and stimulation of HIV-1 integrase by a human homolog of yeast transcription factor SNF5. *Science* 266(5193), 2002-6.**

**Kamata, M., and Aida, Y. (2000). Two putative alpha-helical domains of human immunodeficiency virus type 1 Vpr mediate nuclear localization by at least two mechanisms. *J Virol* 74(15), 7179-86.**

**Kamata, M., Nitahara-Kasahara, Y., Miyamoto, Y., Yoneda, Y., and Aida, Y. (2005). Importin-alpha promotes passage through the nuclear pore**

complex of human immunodeficiency virus type 1 Vpr. *J. Virol.* 79, 3557-64.

Kao, S., Akari, H., Khan, M. A., Dettenhofer, M., Yu, X. F., and Strebel, K. (2003a). Human immunodeficiency virus type 1 Vif is efficiently packaged into virions during productive but not chronic infection. *J Virol* 77(2), 1131-40.

Kao, S., Khan, M. A., Miyagi, E., Plishka, R., Buckler-White, A., and Strebel, K. (2003b). The human immunodeficiency virus type 1 Vif protein reduces intracellular expression and inhibits packaging of APOBEC3G (CEM15), a cellular inhibitor of virus infectivity. *J Virol* 77(21), 11398-407.

Kao, S. Y., Calman, A. F., Luciw, P. A., and Peterlin, B. M. (1987). Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. *Nature* 330(6147), 489-93.

Katz, R. A., Greger, J. G., Boimel, P., and Skalka, A. M. (2003). Human immunodeficiency virus type 1 DNA nuclear import and integration are mitosis independent in cycling cells. *J Virol* 77(24), 13412-7.

Kemler, I., Whittaker, G., and Helenius, A. (1994). Nuclear import of microinjected influenza virus ribonucleoproteins. *Virology* 202(2), 1028-33.

Khiytani, D. K., and Dimmock, N. J. (2002). Characterization of a human immunodeficiency virus type 1 pre-integration complex in which the majority of the cDNA is resistant to DNase I digestion. *Journal of General Virology* 83, 2523-2532.

Kiernan, R. E., Ono, A., Englund, G., and Freed, E. O. (1998). Role of matrix in an early postentry step in the human immunodeficiency virus type 1 life cycle. *J Virol* 72(5), 4116-26.

Kimpton, J., and Emerman, M. (1992). Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated b-galactosidase gene. *J Virol* 66, 2232-2239.

Kleiman, L., and Cen, S. (2004). The tRNALys packaging complex in HIV-1. *Int J Biochem Cell Biol* 36(9), 1776-86.

Kobe, B., Gleichmann, T., Horne, J., Jennings, I. G., Scotney, P. D., and Teh, T. (1999). Turn up the HEAT. *Structure* 7(5), R91-7.

Koepp, D., Wong, D., Corbett, A., and Silver, P. A. (1996). Dynamic localization of the nuclear import receptor and its interactions with transport factors. *J. Cell Biol.* 133, 1163-1176.

Kondo, E., Mammano, F., Cohen, E. A., and Gottlinger, H. G. (1995). The p6gag domain of human immunodeficiency virus type 1 is sufficient for the incorporation of Vpr into heterologous viral particles. *J Virol* 69(5), 2759-64.

**Kotler, M., Simm, M., Zhao, Y. S., Sova, P., Chao, W., Ohnona, S. F., Roller, R., Krachmarov, C., Potash, M. J., and Volsky, D. J. (1997). Human immunodeficiency virus type 1 (HIV-1) protein Vif inhibits the activity of HIV-1 protease in bacteria and in vitro. *J Virol* 71(8), 5774-81.**

**Kuiken, C., Foly, B., Hahn, B., Marx, P., McCutchan, F., Mellors, J., Wolinsky, S. M., and Korber, B. (2001). HIV Sequence Compendium 2001. *Los Alamos National Laboratory.***

**Kukolj, G., Jones, M. S., and Skalka, M. (1997). Subcellular localization of avian sarcoma virus and human immunodefiiency virus type 1 integrase. *J. Virol.* 71, 843-847.**

**Kulkosky, J., Katz, R. A., Merkel, G., and Skalka, A. M. (1995). Activities and substrate specificity of the evolutionarily conserved central domain of retroviral integrase. *Virology* 206(1), 448-56.**

**Kutay, U., Bischoff, F. R., Kostka, S., Kraft, R., and Gorlich, D. (1997). Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor. *Cell* 90(6), 1061-71.**

**LaFemina, R. L., Callahan, P. L., and Cordingley, M. G. (1991). Substrate specificity of recombinant human immunodeficiency virus integrase protein. *J Virol* 65(10), 5624-30.**

**Lam, M. H., Briggs, L. J., Hu, W., Martin, T. J., Gillespie, M. T., and Jans, D. A. (1999). Importin beta recognizes parathyroid hormone-related protein with high affinity and mediates its nuclear import in the absence of importin alpha. *J Biol Chem* 274(11), 7391-8.**

**Le Rouzic, E., Mousnier, A., Rustum, C., Stutz, F., Hallberg, E., Dargemont, C., and Benichou, S. (2002). Docking of HIV-1 Vpr to the nuclear envelope is mediated by the interaction with the nucleoporin hCG1. *J Biol Chem* 277(47), 45091-8.**

**Leavitt, A. D., Robles, G., Alesandro, N., and Varmus, H. E. (1996). Human immunodeficiency virus type 1 integrase mutants retain in vitro integrase activity yet fail to integrate viral DNA efficiently during infection. *J Virol* 70(2), 721-8.**

**Lee, K. K., Haraguchi, T., Lee, R. S., Koujin, T., Hiraoka, Y., and Wilson, K. L. (2001). Distinct functional domains in emerin bind lamin A and DNA-bridging protein BAF. *J Cell Sci* 114(Pt 24), 4567-73.**

**Lee, M. S., and Craigie, R. (1998). A previously unidentified host protein protects retroviral DNA from autointegration. *Proc Natl Acad Sci U S A* 95(4), 1528-33.**

**Lee, S. J., Sekimoto, T., Yamashita, E., Nagoshi, E., Nakagawa, A., Imamoto, N., Yoshimura, M., Sakai, H., Chong, K. T., Tsukihara, T., and Yoneda, Y. (2003). The structure of importin-beta bound to SREBP-2: nuclear import of a transcription factor. *Science* 302(5650), 1571-5.**

**Lee, S. P., Xiao, J., Knutson, J. R., Lewis, M. S., and Han, M. K. (1997). Zn2+ promotes the self-association of human immunodeficiency virus type-1 integrase in vitro. *Biochemistry* 36(1), 173-80.**

**Lewis, P., Hensel, M., and Emerman, M. (1992). Human immunodeficiency virus infection of cells arrested in the cell cycle. *Embo J* 11, 3053-3058.**

**Lewis, P. F., and Emerman, M. (1994). Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J Virol* 68(1), 510-6.**

**Li, G.-R., Simm, M., Potash, M. J., and Volsky, D. J. (1993). Human Immunodeficioency virus type 1 DNA synthesis, integration, and efficient viral replication in growth-arrested T cells. *Journal of Virology* 67, 3969-3977.**

**Limon, A., Devroe, E., Lu, R., Ghory, H. Z., Silver, P. A., and Engelman, A. (2002a). Nuclear localization of human immunodeficiency virus type 1 preintegration complexes (PICs): V165A and R166A are pleiotropic integrase mutants primarily defective for integration, not PIC nuclear import. *J Virol* 76(21), 10598-607.**

**Limon, A., Nakajima, N., Lu, R., Ghory, H. Z., and Engelman, A. (2002b). Wild-type levels of nuclear localization and human immunodeficiency virus type 1 replication in the absence of the central DNA flap. *J Virol* 76(23), 12078-86.**

**Little S, D. G., Schooley R, et al. (2005). Antiretroviral effect of L-000870810, a novel HIV-1 integrase inhibitor, in HIV-1-infected patients (abstract 161). *XII CROI. Boston, MA, February 22-25.***

**Liu, B., Yu, X., Luo, K., Yu, Y., and Yu, X. F. (2004). Influence of primate lentiviral Vif and proteasome inhibitors on human immunodeficiency virus type 1 virion packaging of APOBEC3G. *J Virol* 78(4), 2072-81.**

**Liu, H., Wu, X., Xiao, H., and Kappes, J. C. (1999). Targeting human immunodeficiency virus (HIV) type 2 integrase protein into HIV type 1. *J Virol* 73(10), 8831-6.**

**Llano, M., Delgado, S., Vanegas, M., and Poeschla, E. M. (2004a). Lens epithelium-derived growth factor/p75 prevents proteasomal degradation of HIV-1 integrase. *J Biol Chem* 279(53), 55570-7.**

**Llano, M., S., D., Vanegas, M., and Poeschla, E. M. (2004b). LEDGF/p75 prevents proteasomal degradation of HIV-1 integrase. *J. Biol. Chem.* 279, 33430-6.**

**Llano, M., Vanegas, M., Fregoso, O., Saenz, D., Chung, S., Peretz, M., and Poeschla, E. M. (2004c). LEDGF/p75 determines cellular trafficking of diverse lentiviral but not murine oncoretroviral integrase proteins and is a component of functional lentiviral preintegration complexes. *J Virol* 78(17), 9524-37.**

**Lodi, P. J., Ernst, J. A., Kuszewski, J., Hickman, A. B., Engelman, A., Craigie, R., Clore, G. M., and Gronenborn, A. M. (1995). Solution**

structure of the DNA binding domain of HIV-1 integrase. *Biochemistry* 34(31), 9826-33.

Lombardo, E., Ramirez, J. C., Agbandje-McKenna, M., and Almendral, J. M. (2000). A beta-stranded motif drives capsid protein oligomers of the parvovirus minute virus of mice into the nucleus for viral assembly. *J Virol* 74(8), 3804-14.

Lombardo, E., Ramirez, J. C., Garcia, J., and Almendral, J. M. (2002). Complementary roles of multiple nuclear targeting signals in the capsid proteins of the parvovirus minute virus of mice during assembly and onset of infection. *J Virol* 76(14), 7049-59.

Lu, R., Ghory, H. Z., and Engelman, A. (2005). Genetic analyses of conserved residues in the carboxyl-terminal domain of human immunodeficiency virus type 1 integrase. *J Virol* 79(16), 10356-68.

Lu, R., Limon, A., Devroe, E., Silver, P. A., Cherepanov, P., and Engelman, A. (2004). Class II integrase mutants with changes in putative nuclear localization signals are primarily blocked at a postnuclear entry step of human immunodeficiency virus type 1 replication. *J Virol* 78(23), 12735-46.

Lu, R., Limon, A., Ghory, H. Z., and Engelman, A. (2005). Genetic analyses of DNA-binding mutants in the catalytic core domain of human immunodeficiency virus type 1 integrase. *J Virol* 79(4), 2493-505.

Lu, Y.-L., P. Spearman, and L. Ratner. (1993). Human immunodeficiency virus type 1 viral protein R localization in infected cells and virions. *J. Virol.* 67, 6542-6550.

Luban, J., Bossolt, K. L., Franke, E. K., Kalpana, G. V., and Goff, S. P. (1993). Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. *Cell* 73(6), 1067-78.

Lutzke, R. A., and Plasterk, R. H. (1998). Structure-based mutational analysis of the C-terminal DNA-binding domain of human immunodeficiency virus type 1 integrase: critical residues for protein oligomerization and DNA binding. *J Virol* 72(6), 4841-8.

Lutzke, R. A., Vink, C., and Plasterk, R. H. (1994). Characterization of the minimal DNA-binding domain of the HIV integrase protein. *Nucleic Acids Res* 22(20), 4125-31.

Mabit, H., Breiner, K. M., Knaust, A., Zachmann-Brand, B., and Schaller, H. (2001). Signals for bidirectional nucleocytoplasmic transport in the duck hepatitis B virus capsid protein. *J Virol* 75(4), 1968-77.

Macara, I. G. (2001). Transport into and out of the nucleus. *Microbiol Mol Biol Rev* 65(4), 570-94, table of contents.

Madani, N., and Kabat, D. (2000). Cellular and viral specificities of human immunodeficiency virus type 1 vif protein. *J Virol* 74(13), 5982-7.

Maele, B. V., Rijck, J. D., Claercq, E. D., and Debyser, Z. (2003). Impact of the central polypurine tract on the kinetics of human

immunodeficiency virus type 1 vector transduction. *J. Virol.* 77, 4685-4694.

Maertens, G., Cherepanov, P., Debyser, Z., Engelborghs, Y., and Engelman, A. (2004). Identification and characterization of a functional nuclear localization signal in the HIV-1 integrase interactor LEDGF/p75. *J Biol Chem* 279(32), 33421-9.

Maertens, G., Cherepanov, P., Pluymers, W., Busschots, K., De Clercq, E., Debyser, Z., and Engelborghs, Y. (2003a). LEDGF/p75 is essential for nuclear and chromosomal targeting of HIV-1 integrase in human cells. *J Biol Chem* 278(35), 33528-39.

Mahajan, R., Delphin, C., Guan, T., Gerace, L., and Melchior, F. (1997). A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell* 88(1), 97-107.

Mangeat, B., Turelli, P., Caron, G., Friedli, M., Perrin, L., and Trono, D. (2003). Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. *Nature* 424(6944), 99-103.

Mansharamani, M., Graham, D. R., Monie, D., Lee, K. K., Hildreth, J. E., Siliciano, R. F., and Wilson, K. L. (2003). Barrier-to-autointegration factor BAF binds p55 Gag and matrix and is a host component of human immunodeficiency virus type 1 virions. *J Virol* 77(24), 13084-92.

Margottin, F., Bour, S. P., Durand, H., Selig, L., Benichou, S., Richard, V., Thomas, D., Strebel, K., and Benarous, R. (1998). A novel human WD protein, h-beta TrCp, that interacts with HIV-1 Vpu connects CD4 to the ER degradation pathway through an F-box motif. *Mol Cell* 1(4), 565-74.

Mariani, R., Chen, D., Schrofelbauer, B., Navarro, F., Konig, R., Bollman, B., Munk, C., Nymark-McMahon, H., and Landau, N. R. (2003). Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. *Cell* 114(1), 21-31.

Markosyan, R. M., Cohen, F. S., and Melikyan, G. B. (2003). HIV-1 envelope proteins complete their folding into six-helix bundles immediately after fusion pore formation. *Mol Biol Cell* 14(3), 926-38.

Martin, K., and Helenius, A. (1991). Transport of incoming influenza virus nucleocapsids into the nucleus. *J Virol* 65(1), 232-44.

Masuda, T., Planelles, V., Krogstad, P., and Chen, I. S. (1995). Genetic analysis of human immunodeficiency virus type 1 integrase and the U3 att site: unusual phenotype of mutants in the zinc finger-like domain. *J Virol* 69(11), 6687-96.

Matunis, M. J., Coutavas, E., and Blobel, G. (1996). A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J Cell Biol* 135(6 Pt 1), 1457-70.

**Mazumder, A., Engelman, A., Craigie, R., Fesen, M., and Pommier, Y. (1994). Intermolecular disintegration and intramolecular strand transfer activities of wild-type and mutant HIV-1 integrase. *Nucleic Acids Res* 22(6), 1037-43.**

**Mazumder, A., Neamati, N., Pilon, A. A., Sunder, S., and Pommier, Y. (1996). Chemical trapping of ternary complexes of human immunodeficiency virus type 1 integrase, divalent metal, and DNA substrates containing an abasic site. Implications for the role of lysine 136 in DNA binding. *J Biol Chem* 271(44), 27330-8.**

**McDonald, D., Vodicka, M. A., Lucero, G., Svitkina, T. M., Borisy, G. G., Emerman, M., and Hope, T. J. (2002). Visualization of the intracellular behavior of HIV in living cells. *J Cell Biol* 159(3), 441-52.**

**Melchior, F., and Gerace, L. (1998). Two-way trafficking with Ran. *Trends Cell Biol* 8(5), 175-9.**

**Melikyan, G. B., Markosyan, R. M., Hemmati, H., Delmedico, M. K., Lambert, D. M., and Cohen, F. S. (2000). Evidence that the transition of HIV-1 gp41 into a six-helix bundle, not the bundle configuration, induces membrane fusion. *J Cell Biol* 151(2), 413-23.**

**Mellman, I., and Steinman, R. M. (2001). Dendritic cells: specialized and regulated antigen processing machines. *Cell* 106(3), 255-8.**

**Meltzer, M. S., Skillman. D.R., Gomatos, P. J., Kalter. D.C., and Gendelman, H. E. (1990). Role of mononuclear phagocytes in the pathogenesis of human immunodeficiency virus infection. *Annu Rev Immunol* 8, 169-194.**

**Miller, M. L., Roller, E. E., Zhao, R. Y., Leece, B. A., Ab, O., Baloglu, E., Goldmacher, V. S., and Chari, R. V. (2004). Synthesis of taxoids with improved cytotoxicity and solubility for use in tumor-specific delivery. *J Med Chem* 47(20), 4802-5.**

**Moore, J. D., Yang, J., Truant, R., and Kornbluth, S. (1999). Nuclear import of Cdk/cyclin complexes: identification of distinct mechanisms for import of Cdk2/cyclin E and Cdc2/cyclin B1. *J Cell Biol* 144(2), 213-24.**

**Moore, M. S. (1998). Ran and nuclear transport. *J Biol Chem* 273(36), 22857-60.**

**Moroianu, J., Blobel, G., and Radu, A. (1996). The binding site of karyopherin alpha for karyopherin beta overlaps with a nuclear localization sequence. *Proc Natl Acad Sci U S A* 93(13), 6572-6.**

**Morozov, A., Yung, E., and Kalpana, G. V. (1998). Structure-function analysis of integrase interactor 1/hSNF5L1 reveals differential properties of two repeat motifs present in the highly conserved region. *Proc Natl Acad Sci U S A* 95(3), 1120-5.**

**Mosammaparast, N., and Pemberton, L. F. (2004). Karyopherins: from nuclear-transport mediators to nuclear-function regulators. *Trends Cell Biol* 14(10), 547-56.**

**Mousnier, A., Leh, H., Mouscadet, J. F., and Dargemont, C. (2004). Nuclear import of HIV-1 integrase is inhibited in vitro by styrylquinoline derivatives. *Mol Pharmacol* 66(4), 783-8.**

**Mulder, L. C., Chakrabarti, L. A., and Muesing, M. A. (2002). Interaction of HIV-1 integrase with DNA repair protein hRad18. *J Biol Chem* 277(30), 27489-93.**

**Mumm, S. R., and Grandgenett, D. P. (1991). Defining nucleic acid-binding properties of avian retrovirus integrase by deletion analysis. *J Virol* 65(3), 1160-7.**

**Nachury, M. V., Ryder, U. W., Lamond, A. I., and Weis, K. (1998). Cloning and characterization of hSRP1 gamma, a tissue-specific nuclear transport factor. *Proc Natl Acad Sci U S A* 95(2), 582-7.**

**Nakajima, N., Lu, R., and Engelman, A. (2001). Human immunodeficiency virus type 1 replication in the absence of integrase-mediated dna recombination: definition of permissive and nonpermissive T-cell lines. *J Virol* 75(17), 7944-55.**

**Nakamura, T., Masuda, T., Goto, T., Sano, K., Nakai, M., and Harada, S. (1997). Lack of infectivity of HIV-1 integrase zinc finger-like domain mutant with morphologically normal maturation. *Biochem Biophys Res Commun* 239(3), 715-22.**

**Nakielny, S., and Dreyfuss, G. (1999). Transport of proteins and RNAs in and out of the nucleus. *Cell* 99(7), 677-90.**

**Nguyen, D. H., and Hildreth, J. E. (2000). Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. *J Virol* 74(7), 3264-72.**

**Nie, Z., Bergeron, D., Subbramanian, R. A., Yao, X. J., Checroune, F., Rougeau, N., and Cohen, E. A. (1998). The putative alpha helix 2 of human immunodeficiency virus type 1 Vpr contains a determinant which is responsible for the nuclear translocation of proviral DNA in growth-arrested cells. *J Virol* 72(5), 4104-15.**

**Nishizawa, Y., Usukura, J., Singh, D. P., Chylack, L. T., Jr., and Shinohara, T. (2001). Spatial and temporal dynamics of two alternatively spliced regulatory factors, lens epithelium-derived growth factor (ledgf/p75) and p52, in the nucleus. *Cell Tissue Res* 305(1), 107-14.**

**O'Hara, B. M., and Olson, W. C. (2002). HIV entry inhibitors in clinical development. *Curr Opin Pharmacol* 2(5), 523-8.**

**Ojala, P. M., Sodeik, B., Ebersold, M. W., Kutay, U., and Helenius, A. (2000). Herpes simplex virus type 1 entry into host cells: reconstitution of capsid binding and uncoating at the nuclear pore complex in vitro. *Mol Cell Biol* 20(13), 4922-31.**

O'Neill, R. E., Jaskunas, R., Blobel, G., Palese, P., and Moroianu, J. (1995). Nuclear import of influenza virus RNA can be mediated by viral nucleoprotein and transport factors required for protein import. *J Biol Chem* 270(39), 22701-4.

Orenstein, J. M., Meltzer, M. S., Phipps, T., and Gendelman, H. E. (1988). Cytoplasmic assembly and accumulation of human immunodeficiency virus types 1 and 2 in recombinant human colony-stimulating factor-1-treated human monocytes: an ultrastructural study. *J Virol* 62(8), 2578-86.

Paillart, J. C., and Gottlinger, H. G. (1999). Opposing effects of human immunodeficiency virus type 1 matrix mutations support a myristyl switch model of gag membrane targeting. *J Virol* 73(4), 2604-12.

Pais, G. C., Zhang, X., Marchand, C., Neamati, N., Cowansage, K., Svarovskaia, E. S., Pathak, V. K., Tang, Y., Nicklaus, M., Pommier, Y., and Burke, T. R., Jr. (2002). Structure activity of 3-aryl-1,3-diketo-containing compounds as HIV-1 integrase inhibitors. *J Med Chem* 45(15), 3184-94.

Palmeri, D., and Malim, M. H. (1999). Importin beta can mediate the nuclear import of an arginine-rich nuclear localization signal in the absence of importin alpha. *Mol Cell Biol* 19(2), 1218-25.

Pang, S., Koyanagi, Y., Miles, S., Wiley, C., Vinters, H. V., and Chen, I. S. (1990). High levels of unintegrated HIV-1 DNA in brain tissue of AIDS dementia patients. *Nature* 343(6253), 85-9.

Pannecouque, C., Pluymers, W., Van Maele, B., Tetz, V., Cherepanov, P., De Clercq, E., Witvrouw, M., and Debyser, Z. (2002). New class of HIV integrase inhibitors that block viral replication in cell culture. *Curr Biol* 12(14), 1169-77.

Panther, L. A., Coombs, R. W., Zeh, J. E., Collier, A. C., and Corey, L. (1998). Unintegrated circular HIV-1 DNA in the peripheral mononuclear cells of HIV-1-infected subjects: association with high levels of plasma HIV-1 RNA, rapid decline in CD4 count, and clinical progression to AIDS. *J Acquir Immune Defic Syndr Hum Retrovirol* 17(4), 303-13.

Park, F., and Kay, M. A. (2001). Modified HIV-1 based lentiviral vectors have an effect on viral transduction efficiency and gene expression in vitro and in vivo. *Mol Ther* 4(3), 164-73.

Parolin, C., Taddeo, B., Palu, G., and Sodroski, J. (1996). Use of cis- and trans-acting viral regulatory sequences to improve expression of human immunodeficiency virus vectors in human lymphocytes. *Virology* 222(2), 415-22.

Paxton, W., Connor, R. I., and Landau, N. R. (1993). Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of gag and mutational analysis. *J Virol* 67(12), 7229-37.

**Pemberton, L. F., Blobel, G., and Rosenblum, J. S. (1998). Transport routes through the nuclear pore complex. *Curr Opin Cell Biol* 10(3), 392-9.**

**Peng, W. J., Pan, J. T., Lai, M. C., Chiu, C. F., and Lin, T. H. (1997). The gemone of Moloney murine leukemia virus can be integrated by the integrase of human immunodefiency virus type 1 expressed alone in vivo. *Proc Natl Acad Sci Counc Repub China B* 21, 144-160.**

**Petit, C., Schwartz, O., and Mammano, F. (1999). Oligomerization within virions and subcellular localization of human immunodeficiency virus type 1 integrase. *J Virol* 73(6), 5079-88.**

**Petit, C., Schwartz, O., and Mammano, F. (2000). The karyophilic properties of human immunodeficiency virus type 1 integrase are not required for nuclear import of proviral DNA. *J Virol* 74(15), 7119-26.**

**Pierson, T. C., Zhou, Y., Kieffer, T. L., Ruff, C. T., Buck, C., and Siliciano, R. F. (2002). Molecular characterization of preintegration latency in human immunodeficiency virus type 1 infection. *J Virol* 76(17), 8518-31.**

**Piller, S. C., Caly, L., and Jans, D. A. (2003). Nuclear import of the pre-integration complex (PIC): the Achilles heel of HIV? *Curr Drug Targets* 4(5), 409-29.**

**Pluymers, W., Cherepanov, P., Schols, D., Clercq, E. D., and Debyser, Z. (1999). Nuclear localization of human immunodeficiency virus type 1 integrase expressed as a fusion protein with green fluorescent protein. *Virology* 258, 327-332.**

**Pollard, V. W., and Malim, M. H. (1998). The HIV-1 Rev protein. *Annu Rev Microbiol* 52, 491-532.**

**Pollard, V. W., Michael, W. M., Nakielny, S., Siomi, M. C., Wang, F., and Dreyfuss, G. (1996). A novel receptor-mediated nuclear protein import pathway. *Cell* 86(6), 985-94.**

**Poon, B., and Chen, I. S. (2003). Human immunodeficiency virus type 1 (HIV-1) Vpr enhances expression from unintegrated HIV-1 DNA. *J Virol* 77(7), 3962-72.**

**Popov, S., Rexach, M., Zybarth, G., Reiling, N., Lee, M.-A., Ratner, L., Lane, C. M., Moore, M. S., Blobel, G., and Bukrinsky, M. (1998). Viral protein R regulates nuclear import of the HIV-1 pre-integration complex. *Embo J* 17, 909-917.**

**Potash, M. J., Bentsman, G., Muir, T., Krachmarov, C., Sova, P., and Volsky, D. J. (1998). Peptide inhibitors of HIV-1 protease and viral infection of peripheral blood lymphocytes based on HIV-1 Vif. *Proc Natl Acad Sci U S A* 95(23), 13865-8.**

**Pyhtila, B., and Rexach, M. (2003). A gradient of affinity for the karyopherin Kap95p along the yeast nuclear pore complex. *J Biol Chem* 278(43), 42699-709.**

**Qiu, C., Sawada, K., Zhang, X., and Cheng, X. (2002). The PWWP domain of mammalian DNA methyltransferase Dnmt3b defines a new family of DNA-binding folds.** ***Nat Struct Biol*** **9(3), 217-24.**

**Quillent, C., Borman, A. M., Paulous, S., Dauguet, C., and Clavel, F. (1996). Extensive regions of pol are required for efficient human immunodeficiency virus polyprotein processing and particle maturation.** ***Virology*** **219(1), 29-36.**

**Radu, A., Moore, M. S., and Blobel, G. (1995). The peptide repeat domain of nucleoporin Nup98 functions as a docking site in transport across the nuclear pore complex.** ***Cell*** **81(2), 215-22.**

**Raposo, G., Moore, M., Innes, D., Leijendekker, R., Leigh-Brown, A., Benaroch, P., and Geuze, H. (2002). Human macrophages accumulate HIV-1 particles in MHC II compartments.** ***Traffic*** **3(10), 718-29.**

**Reil, H., Bukovsky, A. A., Gelderblom, H. R., and Gottlinger, H. G. (1998). Efficient HIV-1 replication can occur in the absence of the viral matrix protein.** ***EMBO J*** **17, 2699-2708.**

**Ribbeck, K., and Gorlich, D. (2002). The permeability barrier of nuclear pore complexes appears to operate via hydrophobic exclusion.** ***Embo J*** **21(11), 2664-71.**

**Rice, P., and Mizuuchi, K. (1995). Structure of the bacteriophage Mu transposase core: a common structural motif for DNA transposition and retroviral integration.** ***Cell*** **82(2), 209-20.**

**Roe, T., Reynolds, T. C., Yu, G., and Brown, P. O. (1993). Integration of murine leukemia virus DNA depends on mitosis.** ***Embo J*** **12(5), 2099-108.**

**Ross, E. K., Fuerst, T. R., Orenstein, J. M., O'Neill, T., Martin, M. A., and Venkatesan, S. (1991). Maturation of human immunodeficiency virus particles assembled from the gag precursor protein requires in situ processing by gag-pol protease.** ***AIDS Res Hum Retroviruses*** **7(5), 475-83.**

**Rout, M. P., Aitchison, J. D., Magnasco, M. O., and Chait, B. T. (2003). Virtual gating and nuclear transport: the hole picture.** ***Trends Cell Biol*** **13(12), 622-8.**

**Rout, M. P., Aitchison, J. D., Suprapto, A., Hjertaas, K., Zhao, Y., and Chait, B. T. (2000). The yeast nuclear pore complex: composition, architecture, and transport mechanism.** ***J Cell Biol*** **148(4), 635-51.**

**Ryan, K. J., McCaffery, J. M., and Wente, S. R. (2003). The Ran GTPase cycle is required for yeast nuclear pore complex assembly.** ***J Cell Biol*** **160(7), 1041-53.**

**Saenz, D. T., Loewen, N., Peretz, M., Whitwam, T., Barraza, R., Howell, K. G., Holmes, J. M., Good, M., and Poeschla, E. M. (2004). Unintegrated lentivirus DNA persistence and accessibility to expression in**

nondividing cells: analysis with class I integrase mutants. *J Virol* 78(6), 2906-20.

Saib, A., Puvion-Dutilleul, F., Schmid, M., Peries, J., and de The, H. (1997). Nuclear targeting of incoming human foamy virus Gag proteins involves a centriolar step. *J Virol* 71(2), 1155-61.

Sandefur, S., Smith, R. M., Varthakavi, V., and Spearman, P. (2000). Mapping and characterization of the N-terminal I domain of human immunodeficiency virus type 1 Pr55(Gag). *J Virol* 74(16), 7238-49.

Saphire, A. C., Guan, T., Schirmer, E. C., Nemerow, G. R., and Gerace, L. (2000). Nuclear import of adenovirus DNA in vitro involves the nuclear protein import pathway and hsc70. *J Biol Chem* 275(6), 4298-304.

Schauer, M., and Billich, A. (1992). The N-terminal region of HIV-1 integrase is required for integration activity, but not for DNA-binding. *Biochem Biophys Res Commun* 185(3), 874-80.

Schroder, A. R., Shinn, P., Chen, H., Berry, C., Ecker, J. R., and Bushman, F. (2002). HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* 110(4), 521-9.

Sheehy, A. M., Gaddis, N. C., Choi, J. D., and Malim, M. H. (2002). Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* 418(6898), 646-50.

Sherman, M. P., de Noronha, C. M., Heusch, M. I., Greene, S., and Greene, W. C. (2001). Nucleocytoplasmic shuttling by human immunodeficiency virus type 1 Vpr. *J Virol* 75, 1522-1532.

Shin, C. G., Taddeo, B., Haseltine, W. A., and Farnet, C. M. (1994). Genetic analysis of the human immunodeficiency virus type 1 integrase protein. *J Virol* 68(3), 1633-42.

Shoemaker, C., Hoffman, J., Goff, S. P., and Baltimore, D. (1981). Intramolecular integration within Moloney murine leukemia virus DNA. *J Virol* 40(1), 164-72.

Simon, J. H. M., and Malim, M. H. (1996). The human immunodeficiency virus type 1 Vif protein modulates the postpenetration stability of viral nucleoprotein complexes. *J. Virol.* 70, 5297-5305.

Sirven, A., Pflumio, F., Zennou, V., Titeux, M., Vainchenker, W., Coulombel, L., Dubart-Kupperschmitt, A., and Charneau, P. (2000). The human immunodeficiency virus type-1 central DNA flap is a crucial determinant for lentiviral vector nuclear import and gene transduction of human hematopoietic stem cells. *Blood* 96(13), 4103-10.

Stec, I., Nagl, S. B., van Ommen, G. J., and den Dunnen, J. T. (2000). The PWWP domain: a potential protein-protein interaction domain in nuclear proteins influencing differentiation? *FEBS Lett* 473(1), 1-5.

Stelz, G., Rucker, E., Rosorius, O., Meyer, G., Stauber, R. H., Spatz, M., Eibl, M. M., and Hauber, J. (2002). Identification of two nuclear import signals in the alpha-gene product ICP22 of herpes simplex virus 1. *Virology* 295(2), 360-70.

Stevens, M. P., and Barclay, W. S. (1998). The N-terminal extension of the influenza B virus nucleoprotein is not required for nuclear accumulation or the expression and replication of a model RNA. *J Virol* 72(6), 5307-12.

Stevenson, M., Haggerty, S., Lamonica, C. A., Meier, C. M., Welch, S. K., and Wasiak, A. J. (1990). Integration is not necessary for expression of human immunodeficiency virus type 1 protein products. *J Virol* 64(5), 2421-5.

Stewart, M. (2003). Structural biology. Nuclear trafficking. *Science* 302(5650), 1513-4.

Strebel, K., Daugherty, D., Clouse, K., Cohen, D., Folks, T., and Martin, M. A. (1987). The HIV 'A' (sor) gene product is essential for virus infectivity. *Nature* 328(6132), 728-30.

Strebel, K., Klimkait, T., and Martin, M. A. (1988). A novel gene of HIV-1, vpu, and its 16-kilodalton product. *Science* 241(4870), 1221-3.

Suzuki, Y., and Craigie, R. (2002). Regulatory mechanisms by which barrier-to-autointegration factor blocks autointegration and stimulates intermolecular integration of Moloney murine leukemia virus preintegration complexes. *J Virol* 76(23), 12376-80.

Svarovskaia, E. S., Barr, R., Zhang, X., Pais, G. C., Marchand, C., Pommier, Y., Burke, T. R., Jr., and Pathak, V. K. (2004). Azido-containing diketo acid derivatives inhibit human immunodeficiency virus type 1 integrase in vivo and influence the frequency of deletions at two-long-terminal-repeat-circle junctions. *J Virol* 78(7), 3210-22.

Tan, W., Zhu, K., Segal, D. J., Barbas, C. F., 3rd, and Chow, S. A. (2004). Fusion proteins consisting of human immunodeficiency virus type 1 integrase and the designed polydactyl zinc finger protein E2C direct integration of viral DNA into specific sites. *J Virol* 78(3), 1301-13.

Tasara, T., Maga, G., Hottiger, M. O., and Hubscher, U. (2001). HIV-1 reverse transcriptase and integrase enzymes physically interact and inhibit each other. *FEBS Lett* 507(1), 39-44.

Teo, I., Veryard, C., Barnes, H., An, S. F., Jones, M., Lantos, P. L., Luthert, P., and Shaunak, S. (1997). Circular forms of unintegrated human immunodeficiency virus type 1 DNA and high levels of viral protein expression: association with dementia and multinucleated giant cells in the brains of patients with AIDS. *J Virol* 71(4), 2928-33.

Terwilliger, E. F., Cohen, E. A., Lu, Y. C., Sodroski, J. G., and Haseltine, W. A. (1989). Functional role of human immunodeficiency virus type 1 vpu. *Proc Natl Acad Sci U S A* 86(13), 5163-7.

**Thery, C., Zitvogel, L., and Amigorena, S. (2002). Exosomes: composition, biogenesis and function. *Nat Rev Immunol* 2(8), 569-79.**

**Towers, G. J., Hatziioannou, T., Cowan, S., Goff, S. P., Luban, J., and Bieniasz, P. D. (2003). Cyclophilin A modulates the sensitivity of HIV-1 to host restriction factors. *Nat Med* 9(9), 1138-43.**

**Trotman, L. C., Mosberger, N., Fornerod, M., Stidwill, R. P., and Greber, U. F. (2001). Import of adenovirus DNA involves the nuclear pore complex receptor CAN/Nup214 and histone H1. *Nat Cell Biol* 3(12), 1092-100.**

**Truant, R., and Cullen, B. R. (1999). The arginine-rich domains present in human immunodeficiency virus type 1 Tat and Rev function as direct importin beta-dependent nuclear localization signals. *Mol Cell Biol* 19(2), 1210-7.**

**Tsurutani, N., Kubo, M., Maeda, Y., Ohashi, T., Yamamoto, N., Kannagi, M., and Masuda, T. (2000). Identification of critical amino acid residues in human immunodeficiency virus type 1 IN required for efficient proviral DNA formation at steps prior to integration in dividing and nondividing cells. *J Virol* 74(10), 4795-806.**

**Turlure, F., Devroe, E., Silver, P. A., and Engelman, A. (2004). Human cell proteins and human immunodeficiency virus DNA integration. *Front Biosci* 9, 3187-208.**

**van Gent, D. C., Groeneger, A. A., and Plasterk, R. H. (1992). Mutational analysis of the integrase protein of human immunodeficiency virus type 2. *Proc Natl Acad Sci U S A* 89(20), 9598-602.**

**van Gent, D. C., Vink, C., Groeneger, A. A., and Plasterk, R. H. (1993). Complementation between HIV integrase proteins mutated in different domains. *Embo J* 12(8), 3261-7.**

**Vandegraaff, N., Kumar, R., Hocking, H., Burke, T. R., Jr., Mills, J., Rhodes, D., Burrell, C. J., and Li, P. (2001). Specific inhibition of human immunodeficiency virus type 1 (HIV-1) integration in cell culture: putative inhibitors of HIV-1 integrase. *Antimicrob Agents Chemother* 45(9), 2510-6.**

**Vandekerckhove, L., Christ, F., Van Maele, B., De Rijck, J., Gijsbers, R., Van den Haute, C., Witvrouw, M., and Debyser, Z. (2006). Transient and stable knockdown of the integrase cofactor LEDGF/p75 reveals its role in the replication cycle of human immunodeficiency virus. *J Virol* 80(4), 1886-96.**

**Vanegas, M., Llano, M., Delgado, S., Thompson, D., Peretz, M., and Poeschla, E. (2005). Identification of the LEDGF/p75 HIV-1 integrase-interaction domain and NLS reveals NLS-independent chromatin tethering. *J Cell Sci* 118(Pt 8), 1733-43.**

**Vetter, I. R., Arndt, A., Kutay, U., Gorlich, D., and Wittinghofer, A. (1999). Structural view of the Ran-Importin beta interaction at 2.3 A resolution. *Cell* 97(5), 635-46.**

**Vihinen-Ranta, M., Wang, D., Weichert, W. S., and Parrish, C. R. (2002). The VP1 N-terminal sequence of canine parvovirus affects nuclear transport of capsids and efficient cell infection. *J Virol* 76(4), 1884-91.**

**Vink, C., Oude Groeneger, A. M., and Plasterk, R. H. (1993). Identification of the catalytic and DNA-binding region of the human immunodeficiency virus type I integrase protein. *Nucleic Acids Res* 21(6), 1419-25.**

**Vodicka, M. A. (2001). Determinants for lentiviral infection of non-dividing cells. *Somat Cell Mol Genet* 26(1-6), 35-49.**

**Vodicka, M. A., Koepp, D. M., Silver, P. A., and Emerman, M. (1998). HIV-1 Vpr interacts with the nuclear transport pathway to promote macrophage infection. *Genes Dev* 12, 175-185.**

**Vogt, V. M. (2000). Ubiquitin in retrovirus assembly: actor or bystander? *Proc Natl Acad Sci U S A* 97(24), 12945-7.**

**Wang, J. Y., Ling, H., Yang, W., and Craigie, R. (2001). Structure of a two-domain fragment of HIV-1 integrase: implications for domain organization in the intact protein. *Embo J* 20(24), 7333-43.**

**Wang, P., Palese, P., and O'Neill, R. E. (1997). The NPI-1/NPI-3 (karyopherin alpha) binding site on the influenza a virus nucleoprotein NP is a nonconventional nuclear localization signal. *J Virol* 71(3), 1850-6.**

**Wang, W., Cote, J., Xue, Y., Zhou, S., Khavari, P. A., Biggar, S. R., Muchardt, C., Kalpana, G. V., Goff, S. P., Yaniv, M., Workman, J. L., and Crabtree, G. R. (1996). Purification and biochemical heterogeneity of the mammalian SWI-SNF complex. *Embo J* 15(19), 5370-82.**

**Weinberg, J. B., Matthews, T.J., Cullen, B.R., and Malim, M.H. (1991). Productive human immunodeficiency virus type 1 (HIV-1) infection of nonproliferating human monocytes. *J. Exp. Med.* 174, 1477-1482.**

**Weis, K. (2003). Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle. *Cell* 112(4), 441-51.**

**Weis, K., Ryder, U., and Lamond, A. I. (1996). The conserved amino-terminal domain of hSRP1 alpha is essential for nuclear protein import. *Embo J* 15(8), 1818-25.**

**Wiese, C., Wilde, A., Moore, M. S., Adam, S. A., Merdes, A., and Zheng, Y. (2001). Role of importin-beta in coupling Ran to downstream targets in microtubule assembly. *Science* 291(5504), 653-6.**

**Wills, J. W., and Craven, R. C. (1991). Form, function, and use of retroviral gag proteins. *Aids* 5(6), 639-54.**

**Wiskerchen, M., and Muesing, M. A. (1995). Human immunodeficiency virus type 1 integrase: effects of mutations on viral ability to integrate, direct viral gene expression from unintegrated viral DNA templates, and sustain viral propagation in primary cells. *J Virol* 69(1), 376-86.**

**Wisnivesky, J. P., Leopold, P. L., and Crystal, R. G. (1999). Specific binding of the adenovirus capsid to the nuclear envelope. *Hum Gene Ther* 10(13), 2187-95.**

**Witvrouw, M., Pannecouque, C., Switzer, W. M., Folks, T. M., De Clercq, E., and Heneine, W. (2004). Susceptibility of HIV-2, SIV and SHIV to various anti-HIV-1 compounds: implications for treatment and postexposure prophylaxis. *Antivir Ther* 9(1), 57-65.**

**Woerner, A. M., and Marcus-Sekura, C. J. (1993). Characterization of a DNA binding domain in the C-terminus of HIV-1 integrase by deletion mutagenesis. *Nucleic Acids Res* 21(15), 3507-11.**

**Wu, X., Liu, H., Xiao, H., Conway, J. A., Hehl, E., Kalpana, G. V., Prasad, V., and Kappes, J. C. (1999). Human immunodeficiency virus type 1 integrase protein promotes reverse transcription through specific interactions with the nucleoprotein reverse transcription complex. *J Virol* 73(3), 2126-35.**

**Wu, X., Liu, H., Xiao, H., Conway, J. A., Hunter, E., and Kappes, J. C. (1997). Functional RT and IN incorporated into HIV-1 particles independently of the Gag/Pol precursor protein. *Embo J* 16(16), 5113-22.**

**Wu, X., Wakefield, J. K., Liu, H., and Kappes, J. C. (2001). Analysis of lenti- and trans-lentiviral vector genetic recombination. *Dev Biol (Basel)* 106, 237-48; discussion 249, 253-63.**

**Wu, Y. (2004). HIV-1 gene expression: lessons from provirus and non-integrated DNA. *Retrovirology* 1(1), 13.**

**Wu, Y., and Marsh, J. W. (2001). Selective transcription and modulation of resting T cell activity by preintegrated HIV DNA. *Science* 293(5534), 1503-6.**

**Wu, Y., and Marsh, J. W. (2003). Gene transcription in HIV infection. *Microbes Infect* 5(11), 1023-7.**

**Xiao, Z., Liu, X., and Lodish, H. F. (2000). importin beta mediates nuclear translocation of Smad 3. *J. Biol. Chem.* 275, 23425-8.**

**Yamaguchi, R., and Newport, J. (2003). A role for Ran-GTP and Crm1 in blocking re-replication. *Cell* 113(1), 115-25.**

**Yamashita, M. and Emerman M.(2004). Capsid is a dominant determinant of retrovirus infectivity in nondividing cells.*J. Virol.*78(11), 5670-78.**

**Yamashita, M. and Emerman M.(2005). The cell cycle independence of HIV infections is not determined by known karyophilic viral elements. *PLoS Pathogens.*1(3),0170-0177.**

**Yamashita, M. and Emerman M.(2006). Retroviral infection of non-dividing cells: old and new perspectives. *virology* 344,88-93.**

**Yao, X. J., Kobinger, G., Dandache, S., Rougeau, N., and Cohen, E. (1999). HIV-1 Vpr-chloramphenicol acetyltransferase fusion proteins: sequence requirement for virion incorporation and analysis of antiviral effect. *Gene Ther* 6(9), 1590-9.**

**Yao, X. J., Mouland, A. J., Subbramanian, R. A., Forget, J., Rougeau, N., Bergeron, D., and Cohen, E. A. (1998). Vpr stimulates viral expression and induces cell killing in human immunodeficiency virus type 1-infected dividing Jurkat T cells. *J Virol* 72(6), 4686-93.**

**Yao, X. J., Subbramanian, R. A., Rougeau, N., Boisvert, F., Bergeron, D., and Cohen, E. A. (1995). Mutagenic analysis of human immunodeficiency virus type 1 Vpr: role of a predicted N-terminal alpha-helical structure in Vpr nuclear localization and virion incorporation. *J Virol* 69(11), 7032-44.**

**Young, S. D. (2001). Inhibition of HIV-1 integrase by small molecules: the potential for a new class of AIDS chemotherapeutics. *Curr Opin Drug Discov Devel* 4(4), 402-10.**

**Yuan, B., Fassati, A., Yueh, A., and Goff, S. P. (2002). Characterization of Moloney murine leukemia virus p12 mutants blocked during early events of infection. *J Virol* 76(21), 10801-10.**

**Yung, E., Sorin, M., Pal, A., Craig, E., Morozov, A., Delattre, O., Kappes, J., Ott, D., and Kalpana, G. V. (2001). Inhibition of HIV-1 virion production by a transdominant mutant of integrase interactor 1. *Nat Med* 7(8), 920-6.**

**Yung, E., Sorin, M., Wang, E. J., Perumal, S., Ott, D., and Kalpana, G. V. (2004). Specificity of interaction of INI1/hSNF5 with retroviral integrases and its functional significance. *J Virol* 78(5), 2222-31.**

**Zennou, V., Petit, C., Guetard, D., Nerhbass, U., Montagnier, L., and Charneau, P. (2000). HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* 101(2), 173-85.**

**Zennou, V., Serguera, C., Sarkis, C., Colin, P., Perret, E., Mallet, J., and Charneau, P. (2001). The HIV-1 DNA flap stimulates HIV vector-mediated cell transduction in the brain. *Nat Biotechnol* 19(5), 446-50.**

**Zhang, H., Dornadula, G., Orenstein, J., and Pomerantz, R. J. (2000). Morphologic changes in human immunodeficiency virus type 1 virions secondary to intravirion reverse transcription: evidence indicating that reverse transcription may not take place within the intact viral core. *J Hum Virol* 3(3), 165-72.**

**Zhang, H., Yang, B., Pomerantz, R. J., Zhang, C., Arunachalam, S. C., and Gao, L. (2003). The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. *Nature* 424(6944), 94-8.**

**Zheng, R., Ghirlando, R., Lee, M. S., Mizuuchi, K., Krause, M., and Craigie, R. (2000). Barrier-to-autointegration factor (BAF) bridges DNA in a discrete, higher-order nucleoprotein complex. *Proc Natl Acad Sci U S A* 97(16), 8997-9002.**

**Zheng, R., Jenkins, T. M., and Craigie, R. (1996). Zinc folds the N-terminal domain of HIV-1 integrase, promotes multimerization, and enhances catalytic activity. *Proc Natl Acad Sci U S A* 93(24), 13659-64.**

**Zhou, Y., Lu, Y., and Ratner, L. (1998). Arginine residues in the C-terminus of HIV-1 Vpr are important for nuclear localization and cell cycle arrest. *Virology* 242(2), 414-24.**

**Zhu, K., Dobard, C., and Chow, S. A. (2004). Requirement for integrase during reverse transcription of human immunodeficiency virus type 1 and the effect of cysteine mutations of integrase on its interactions with reverse transcriptase. *J Virol* 78(10), 5045-55.**

**Zielske, S. P., and Stevenson, M. (2005). Importin 7 may be dispensible for human immunodeficiency virus type 1 and simian immunodeficiency virus infection of primary macrophages. *J. Virol.* 79, 11541-6.**

**Zouhiri, F., Mouscadet, J. F., Mekouar, K., Desmaele, D., Savoure, D., Leh, H., Subra, F., Le Bret, M., Auclair, C., and d'Angelo, J. (2000). Structure-activity relationships and binding mode of styrylquinolines as potent inhibitors of HIV-1 integrase and replication of HIV-1 in cell culture. *J Med Chem* 43(8), 1533-40.**

# APPENDIX I

JOURNAL OF VIROLOGY, Mar. 2004, p. 3170–3177 Vol. 78, No. 6
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.6.3170–3177.2004


# Assessment of the Role of the Central DNA Flap in Human Immunodeficiency Virus Type 1 Replication by Using a Single-Cycle Replication System

Zhujun Ao, Xiaojian Yao,* and Éric A. Cohen*

*Laboratoire de rétrovirologie humaine, Département de microbiologie et immunologie, Faculté de médecine, Université de Montréal, Montréal, Québec, Canada H3C 3J7*

Received 17 November 2003/Accepted 24 November 2003

**In this study, reverse transcriptase (RT)- and integrase (IN)-defective human immunodeficiency virus type 1 (HIV-1) was transcomplemented with Vpr-RT-IN fusion proteins to delineate *pol* sequences important for HIV-1 replication. Our results reveal that a 194-bp sequence encompassing the 3′end of the IN gene and containing the central DNA flap is necessary and sufficient for efficient HIV-1 single-cycle replication in dividing and nondividing cells. Furthermore, we show that the central DNA flap enhances HIV-1 single-round replication by five- to sevenfold, primarily by facilitating nuclear import of proviral DNA. In agreement with previous reports, our data support a functional role of the central DNA flap during the early stages of HIV-1 infection.**

The complexity of human immunodeficiency virus type 1 (HIV-1) replication is attributed in large part to the intricate interplay that takes place between *cis*-acting sequences present on viral nucleic acids and viral or host cell proteins that function in *trans* (22). The HIV-1 *pol* gene encodes three enzymatic proteins, including protease, reverse transcriptase (RT), and integrase (IN), which play critical roles during specific stages of the virus infection cycle. Soon after virus entry, RT catalyzes the conversion of the viral RNA genome into double-stranded proviral DNA, while IN mediates proviral DNA integration into the host cell genome (for a review, see references 10, 15, and 21). Even though extensive in vitro biochemical and mechanistic studies have greatly contributed to a better understanding of the primary function and mode of action of RT and IN enzymes, studies performed in the context of HIV-1 infectious proviral clones have revealed that some mutations and/or internal deletions in RT or IN can significantly alter steps other than reverse transcription or integration, such as virus assembly or release, or even inactivate virus infectivity (1, 2, 4, 14, 20, 35). These pleiotropic phenotypes resulting from mutagenic analysis suggest that RT and IN may play other roles which are independent of their enzymatic activities. Furthermore, introduction of mutations in RT and/or IN sequences may simultaneously affect a *cis*-acting element(s) present within the *pol* gene sequence that is required for efficient virus replication.

In contrast to oncoretroviruses, HIV-1 and other lentiviruses have the capacity to infect nondividing cell populations, such as macrophages, mucosal dendritic cells, and nondividing T cells, since they do not depend on host cell mitosis to mediate the nuclear translocation of their preintegration complex (PIC) (5, 12, 27, 28, 31, 40). At the molecular level, HIV-1 PIC nuclear transport was shown to proceed through intact nuclear pore complexes by an active and energy-dependent mechanism that involves the karyophilic properties of several PIC-associated viral proteins, including Matrix ($MAp17^{gag}$), IN, and Vpr (3, 6, 12, 19, 20, 23, 24, 32, 39). In addition, a *cis*-acting element designated the central DNA flap located in the 3′ region of the *pol* gene sequence was also shown to contribute to the nuclear import of HIV-1 proviral DNA in both dividing and nondividing cells (45). The central DNA flap is a region of triple-stranded DNA created by two discrete half-genomic fragments with a central strand displacement event controlled in *cis* by a central polypurine tract (cPPT) and a central termination sequence (CTS) during HIV-1 reverse transcription (7, 8). HIV-1 viruses carrying an inactivated cPPT or CTS were reported to exhibit a considerable impairment of viral replication in different dividing and nondividing target cells (7, 8), presumably because of a defect at the level of the nuclear import of the PIC (45). However, these results have been put into question recently by two other studies (13, 29), which provided evidence indicating that the central DNA flap did not play a major role in either PIC nuclear import or HIV-1 replication in a variety of cell lines. Interestingly, in contrast to these studies that used replication-competent viruses, numerous other studies have reported that the central DNA flap conferred a transduction advantage of approximately 2- to 10-fold on HIV-1-derived lentiviral vectors, thus suggesting that the central DNA flap facilitated an early step(s) in lentiviral infection (11, 17, 30, 33, 34, 37, 46). The exact impact of the central DNA flap on the early steps of HIV-1 infection is still an open question that remains to be clarified.

**Requirement of *pol* gene sequence for efficient HIV-1 single-cycle infection.** In order to investigate the impact of the HIV-1 *pol* gene sequence on virus replication, we generated a previously described HIV-1 RT and IN transcomplemented replication system (16, 41). An RT- and IN-defective HIV-1 provirus ($R^-/RI^-$) with an intact *pol* gene sequence was con-

* Corresponding author. Mailing address: University of Montreal, Microbiology and Immunology, CP 6128, Station Centre-Ville, Montreal, PQ, H3C 3J7 Canada. Phone, fax, and e-mail for Éric A. Cohen: Phone: (514) 343-5967. Fax: (514) 343-5995. E-mail: [redacted]. Phone, fax, and e-mail for Xiaojian Yao: Phone: (514) 343-6280. Fax: (514) 343-5985. E-mail: [redacted].

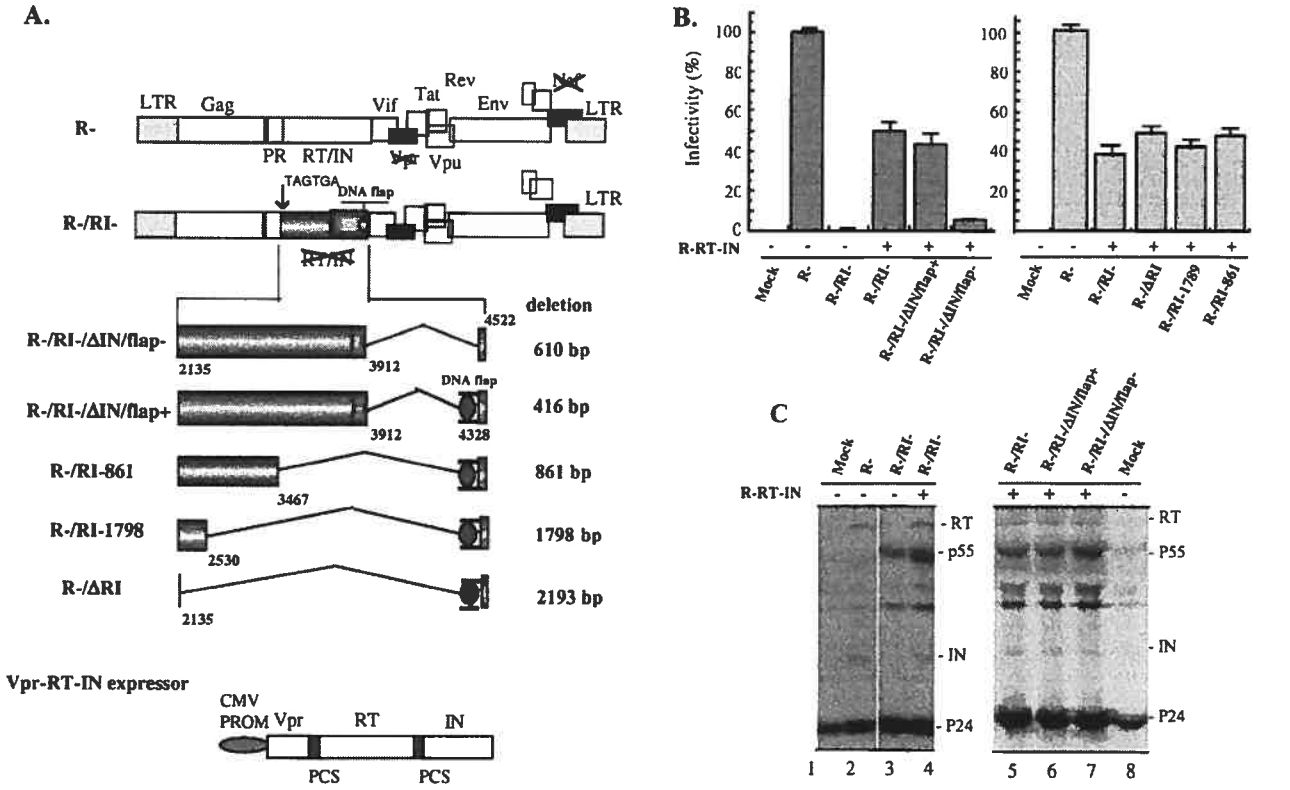


FIG. 1. Effect of the HIV-1 IN and RT gene sequences on the infectivity of RT/IN-transcomplemented virus. (A) Schematic structure of HIV-1 proviruses carrying a mutation and/or deletions in *pol* and of the plasmid encoding the Vpr-RT-IN fusion protein. Provirus $R^-/RI^-$ was constructed by replacing the first two amino acids of RT with two premature stop codons (TGA TAG) in $HxBruR^-(R^-)$ provirus. In $R^-/RI^-/\Delta IN/flap^-$ provirus, a 610-bp fragment of the IN gene sequence (including cPPT/CTS) was deleted. The $R^-/RI^-/\Delta IN/flap^+$, $R^-/RI$-861, $R^-/RI$-1798, and $R^-/\Delta RI$ proviruses harbor different deletions within the RT and/or IN gene sequences but contain the 194-bp sequence in the 3′-end region of IN, which harbors the cPPT/CTS *cis*-acting elements. PR, protease. (B) The infectivity of trans-complemented virus produced in 293T cells was evaluated by MAGI assay. Equal amounts (15 ng of $p24^{gag}$ antigen) of the different viruses were used to infect HeLa-CD4-β-Gal cells, and the number of infected cells was monitored by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining. The infectivity (% infectivity) of each virus stock was calculated as the ratio of the number of β-Gal-positive cells relative to the number of β-Gal-positive cells obtained with the wt virus ($R^-$). The number of β-Gal-positive cells detected with the $R^-$ virus ranged between 330 to 410 and was set at 100%. The results are representative of three independent experiments. (C) To evaluate Vpr-mediated transincorporation of RT and IN in viral particles, radiolabeled viruses were isolated from cell supernatants, lysed, immunoprecipitated with anti-HIV antibodies, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12.5% acrylamide). LTR, long terminal repeat.

structed by replacing the first two amino acids of RT with two premature stop codons (TGA TAG) in a Vpr- and Nef-defective HxBc2-derived HIV-1 provirus ($R^-$) by using a two-step PCR-based method (44). To transcomplement the RT and IN defect of HIV-1 virus, a Vpr-RT-IN fusion protein expression plasmid (CMV-R-RT-IN) was also made by inserting a PCR-amplified HIV-1 RT and IN gene cDNA in frame with Vpr into the SVCMV-Vpr plasmid (44) (Fig. 1A). It has been shown that HIV-1 RT and IN enzymatic defects can be restored in *trans* through Vpr-mediated virion incorporation of Vpr-RT-IN fusion protein, leading to production of viral particles that can undergo a single-round replication (16, 41). To test the infectivity of the transcomplemented RT- and IN-defective virus, 293T cells were transfected with $R^-/RI^-$ provirus or cotransfected with CMV-R-RT-IN plasmid by using the calcium phosphate DNA precipitation method (42). In parallel, the wild-type (wt) provirus ($R^-$) was used as a positive control. At 48-h posttransfection, virus stocks were generated from supernatants and quantitated by p24 measurements by using an HIV-1 p24 enzyme-linked immunosorbent assay (ELISA) kit (AIDS Vaccine Program of the Frederick Cancer Research and Development Center) as described previously (43). Then the infectivity of each virus was examined by infecting HeLa-CD4-β-galactosidase (HeLa-CD4-[β-Gal]) cells with equal amounts (15 ng of $p24^{gag}$ antigen/well) of virus and evaluated by MAGI assay 48-h postinfection (p.i.), as described previously (26). Consistent with a previous report (41), RT- and IN-defective $R^-/RI^-$ viruses were found to be infectious by MAGI assay only when they were transcomplemented with RT and IN during viral production, reaching infectivity levels corresponding to 40 to 50% of the wt level ($R^-$ virus) (Fig. 1B, left panel). To test the efficiency of Vpr-mediated RT and IN transincorporation, transfected 293T cells were radiolabeled and the resulting viral particles were analyzed for viral protein content by immunoprecipitation by using anti-HIV-1 serum (42). Results reveal that, as expected, the $R^-/RI^-$ viral particles did not contain any RT or IN proteins (Fig. 1C, lane 3), whereas the transcomplemented $R^-/RI^-$ virus incorporated RT and IN proteins at levels comparable to those for the wt $R^-$ virus (Fig. 1C, compare lane 4 to lane 2). Interestingly,

it was noted that significant amounts of unprocessed $Pr55^{gag}$ accumulated in $R^-/RI^-$ viral particles (Fig. 1C, compare lanes 3 and 4 to lane 2). This maturation defect is likely to result from an impairment of protease activation, given that the Gag-Pol polyprotein precursor was truncated by early termination of RT and IN and is believed to be responsible for the observed 50 to 60% reduction of virus infectivity (Fig. 1B).

We next analyzed the requirement of RT and IN gene sequences for virus replication by using this Vpr-RT-IN transcomplementation system. A series of proviruses with deletions of the RT and/or IN gene derived from the $R^-/RI^-$ provirus were constructed as indicated in Fig. 1A. In the $R^-/RI^-/\Delta IN/flap^-$ provirus, a 610-bp sequence encompassing a large part of the IN gene and including the cPPT and CTS *cis*-acting sequences (from nucleotide 3,912 to 4,522; +1 corresponds to the transcription initiation site of the BRU strain), which were previously shown to play an important role in HIV-1 replication, were deleted (7, 8, 11). In the $R^-/RI^-/\Delta IN/flap^+$ provirus, a smaller deletion of 494 bp was introduced, thus leaving intact a 194-bp sequence, including cPPT/CTS elements at the 3′ end of the IN gene. To further test the impact of the RT gene sequence on virus replication, different regions of the RT gene sequence were further deleted, based on the $R^-/RI^-/\Delta IN/flap^+$ provirus and designated $R^-/RI$-861, $R^-/RI$-1798, and $R^-/\Delta RI$ (Fig. 1A). In the $R^-/\Delta RI$ provirus, all the RT and IN gene sequences except the 194 bp containing the cPPT/CTS sequences were deleted (a deletion of 2,193 bp). The infectivity of each transcomplemented virus with a deletion of the RT and/or IN gene was analyzed by MAGI assay. Deletion of the 3′ region of IN encompassing the cPPT/CTS elements resulted in a substantial five- to sevenfold decrease of viral infectivity compared to that of the transcomplemented $R^-RI^-$ virus (Fig. 1B, left panel). This sharp decrease in viral infectivity was not due to variation in the levels of RT and/or IN transincorporated into viral particles, since they were found to be similar in both transcomplemented viruses (Fig. 1C, compare lanes 7 and 5). In contrast, maintenance of a 194-bp sequence in the 3′ region of the IN gene sequence, which includes the cPPT/CTS elements ($R^-/RI^-/\Delta IN/flap^+$), restored infectivity to a level similar to that of the transcomplemented $R^-/RI^-$ virus (Fig. 1B, left panel). Interestingly, deletion of RT gene sequences ($R^-/RI$-861, $R^-/RI$-1798, and $R^-/\Delta RI$) had no impact on viral infectivity as long as the 194-bp sequence in the 3′ end region of IN was intact (Fig. 1B, right panel). Furthermore, maintenance of this 194-bp fragment was found to confer a six- to sevenfold infectivity advantage to single-cycle replicating virus in dividing and aphidicolin growth-arrested C8166 T cells (data not shown). Overall, these results indicate that the 3′ region of the IN gene sequence harbors *cis*-acting determinants(s) that substantially enhance the replication of HIV-1 toward dividing and nondividing $CD4^+$ T cells in the context of a single-cycle infection system, while other RT and IN gene sequences are clearly dispensable.

**The cPPT contributes to efficient HIV-1 single-cycle replication.** To further confirm that *cis*-acting element(s) in the 3′ region of the IN gene sequence contribute to efficient single-cycle replication, we introduced a 10-bp substitution in the cPPT element in the $R^-/\Delta RI$ provirus and generated a cPPT-defective mutant designated $R^-/\Delta RI/cPPT^-$ (Fig. 2A). These specific mutations in the cPPT element have previously been reported to prevent the formation of the central DNA flap during reverse transcription (13, 29, 45). Prior to testing virus infectivity, the levels of transincorporated RT and IN in both the cPPT mutant and the control virus were examined by radiolabeling and immunoprecipitation as described in Fig. 1. Similar levels of virion-associated RT, IN, $p24^{gag}$, and $p55^{gag}$ were detected in transcomplemented $R^-/RI^-$, $R^-/\Delta RI$, and $R^-/\Delta RI/cPPT^-$ virus preparations (Fig. 2B). To compare the replication potential of transcomplemented $R^-/\Delta RI/cPPT^-$ and $R^-/\Delta RI$ viruses, $CD4^+$ MT4 cells and phytohemagglutinin-stimulated human peripheral blood mononuclear cells (h-PBMCs) were infected with equal amounts of each virus stock for 8 h, and at different time intervals, virion-associated $p24^{gag}$ antigen levels in the supernatant were measured by anti-p24 ELISA. Disruption of the cPPT was found to decrease by five- to sevenfold viral replication in both MT4 T cells and activated h-PBMCs compared to that in the trans-complemented $R^-/\Delta RI$ control virus (Fig. 2C and D). Hence, we conclude that the central DNA flap is the necessary determinant in the 3′ region of the IN gene sequence that contributes to efficient single-cycle virus replication.

**Effect of the central DNA flap on late-reverse-transcribed DNA products, viral cDNA nuclear import, and proviral DNA integration in h-PBMCs.** To investigate the mechanism(s) underlying the action of the central DNA flap during single-cycle replication, we first analyzed the efficiency of proviral DNA integration in h-PBMCs infected with $cPPT^+$ or $cPPT^-$ transcomplemented viruses by using a previously described, sensitive, two-step Alu-PCR technique (9). Results reveal that levels of integrated proviral DNA detected in the $R^-/\Delta RI/cPPT^-$ sample were five- to sevenfold lower than those detected in the $R^-/\Delta RI$ sample at both 24- and 36-h p.i. (Fig. 3A and B). Interestingly, this significant decrease in proviral DNA integration correlated well with the five- to sevenfold replication defect observed with the cPPT-defective virus, indicating that the central DNA flap contributes to efficient viral single-cycle replication by acting on an early stage(s) of viral replication at and/or prior to viral integration. To further investigate at which early step(s) of the infection cycle the central DNA flap acts, we analyzed the total amounts of viral cDNA present at different early time points by PCR following infection of h-PBMCs with equal amounts of $R^-/\Delta RI$ or $R^-/\Delta RI/cPPT^-$ viruses. Briefly, an equal number ($2 \times 10^6$ cells) of h-PBMCs were lysed in lysis buffer (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.05% NP-40, 0.05% Tween 20) and treated with proteinase K (100 μg/ml) prior to phenol-chloroform DNA purification. Then fivefold-serially diluted DNA samples were subjected to PCR analysis with specific primers (5′-U3, 5′-GGA TGGTGCTTCAAGCTAGTACC-3′, and 3′-Gag, 5′-ACTGA CGCTCTCGCACCCATCTCTCTC-3′) and further analyzed by Southern blotting by using specific PCR DIG-Labeling probes (Roche Diagnostics, Laval, Quebec, Canada). As shown in Fig. 4A, at 6-h p.i., similar amounts of total viral cDNA were detected in $R^-/\Delta RI$- and $R^-/\Delta RI/cPPT^-$-infected cells (4.9 copies/cell versus 4.6 viral copies/cell), suggesting that both transcomplemented viruses entered cells with similar efficiencies and underwent uncoating and reverse transcription at comparable rates. In contrast, between 12- and 24-h p.i., total amounts of late $cPPT^-$ reverse-transcribed products decreased at a rate that was clearly different from that of viral $cPPT^+$

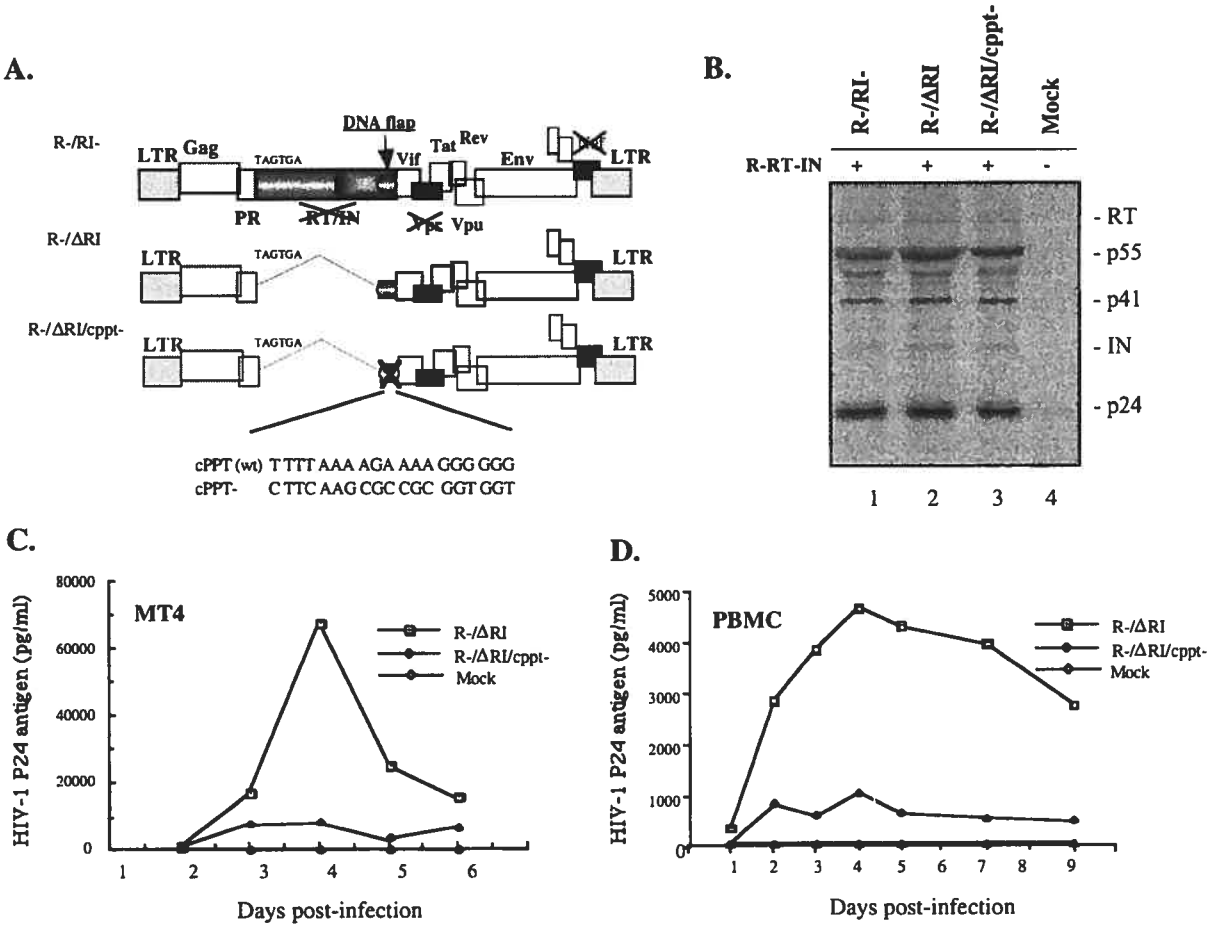


FIG. 2. The central DNA flap contributes to efficient HIV-1 single-cycle replication. (A) Schematic structure of HIV-1 provirus with the RT/IN gene deleted ($R^-/\Delta RI$) and the cPPT$^-$ mutant ($R^-/\Delta RI/cPPT^-$). The cPPT element was inactivated by introduction of ten nucleotide substitution mutations, as indicated. (B) To evaluate RT and IN transincorporation, [$^{35}$S]-methionine-radiolabeled viruses were collected from transfected 293T cells, lysed, and analyzed by immunoprecipitation by using anti-HIV antibodies. To test the replication potential of each virus stock, CD4$^+$ MT4 T cells (C) or PHA-stimulated human PBMCs (D) were infected with equal amounts of $R^-/\Delta RI$ or $R^-/\Delta RI/cPPT^-$ viruses. At different time intervals after infection, viral production was monitored in the supernatants by measurement of HIV-1 $p24^{gag}$ antigen by using a p24 ELISA assay. The results are representative of two independent experiments.

cDNA products. At 12-h and 24-h p.i, levels of viral cPPT$^-$ cDNA were reduced by approximately 45 and 40% compared to the levels of cPPT$^+$ cDNA, which stayed quite stable during the same time interval (between approximately 87 and 88% of their levels at 6 h) (Fig. 4A). At later time points (between 24 and 48 h), both cPPT$^+$ and cPPT$^-$ viral cDNAs decreased at similar rates, most probably as a result of the dilution of unintegrated viral cDNA that occurs upon cell division. This difference in the rate of viral cDNA decrease detected between $R^-/\Delta RI$ and $R^-/\Delta RI/cPPT^-$ infection was not due to intrinsic variation between samples, since similar levels of a control cellular DNA (human β-2-adrenergic receptor [β2-AR] gene) were detected by PCR in each sample (Fig. 4B, left panel). These results suggest that the central DNA flap does not interfere with the rate of the reverse transcription step per se but appears to influence the rate of accumulation of total viral cDNA product.

In parallel, we analyzed viral cDNA nuclear import by subcellular fractionation and subsequent detection of viral cDNA associated with nuclear or cytoplasmic fractions as previously described (36). Human PBMCs were infected with equivalent amounts of transcomplemented $R^-/\Delta RI/cPPT^-$ or $R^-/\Delta RI$ viruses, and cytoplasmic and nuclear fractions were isolated from the same number of cells at 24-h p.i. All fractions were then analyzed by PCR as described in the legend to Fig. 4A. The presence of total viral DNA was visualized by ethidium bromide staining, and the staining intensity of each amplified DNA product was quantified by using a ChemiImager 5500 system with AlphaEaseFC software (Alpha Innotech Corporation). The results shown in Fig. 4C (upper panel) reveal that at 24-h p.i. total amounts of viral cDNA in the $R^-/\Delta RI$-infected sample (including cytoplasmic and nuclear fractions) were approximately threefold higher than those detected in the $R^-/\Delta RI/cPPT^-$ infected sample, thus confirming the data obtained from Fig. 4A. Interestingly, while approximately 75% of total viral cDNA was detected in the nuclear fraction of $R^-/\Delta RI$-infected cells, only 30% of total viral cDNA was found in the nuclei of $R^-/\Delta RI/cPPT^-$-infected cells (Fig. 4C, upper

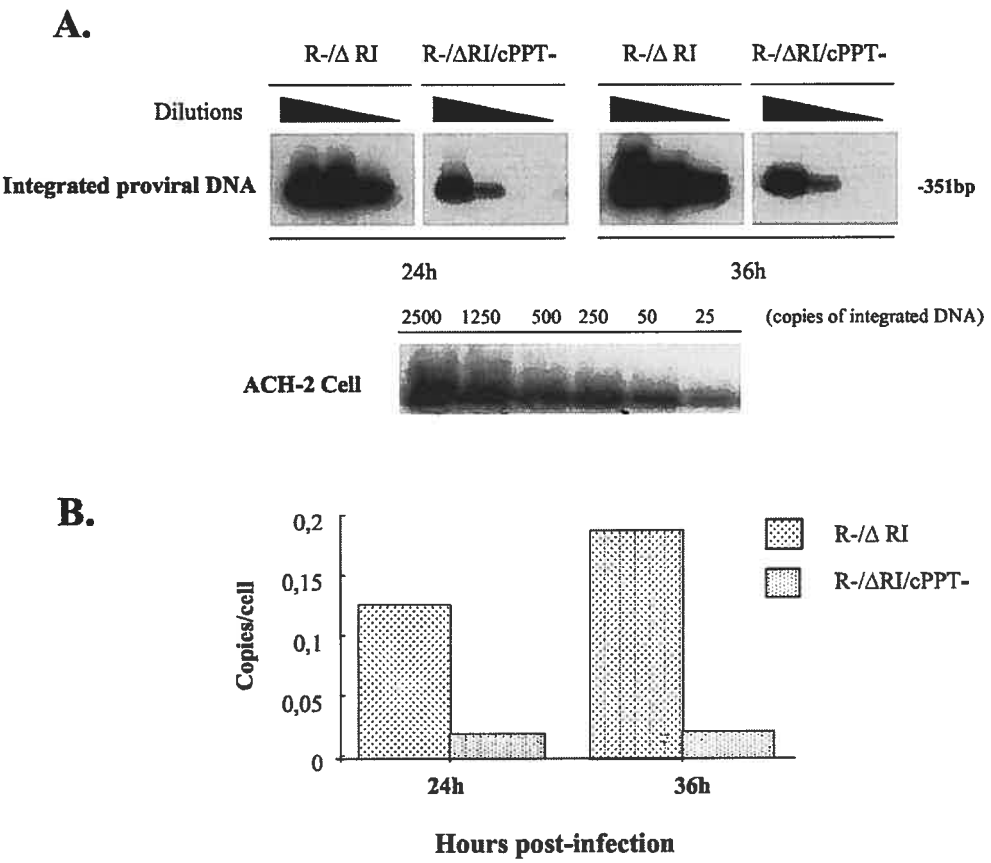


FIG. 3. Effect of the central DNA flap on HIV-1 proviral DNA integration in h-PBMC. (A) h-PBMCs were infected with $R^-/\Delta RI$ or $R^-/\Delta RI/cPPT^-$ virus (125 ng of p24/$10^6$ cells). At 24- and 36-h p.i., the cells were lysed, and serially diluted cell lysates were analyzed by two-step Alu-PCR and Southern blotting for specific detection of integrated proviral DNA from infected PBMCs (upper panel) or from ACH-2 cells as the quantitative control (lower panel). (B) Quantitative analysis of integrated proviral DNA in single-cycle infection. The bands in panel A were quantified by laser densitometry, and the number of integrated proviral DNA copies per cell was determined by using the PCR-generated standard curve derived from ACH-2 cells.

panel). Moreover, the absolute levels of nuclear-associated viral cDNA were approximately sevenfold higher with the wt virus than with the cPPT-defective virus. The integrity of the fractionation procedure was validated by detection of mitochondrial DNA and β-globin DNA, as described previously (18, 36, 38). Results showed that mitochondrial and β-globin DNAs were found solely in the cytoplasm and the nucleus, respectively (Fig. 4C, lower panel). In addition, levels of each of these control cellular DNAs were similar in both $R^-/\Delta RI$ and $R^-/\Delta RI/cPPT^-$ subcellular fractions, confirming that equivalent amounts of nuclear and cyctoplasmic fractions were analyzed.

Several recent studies have investigated the role of the central DNA flap in HIV-1 replication and nuclear import and have reached conflicting conclusions (11, 13, 17, 29, 30, 33, 34, 37, 45, 46). In this study, we reexamined this question by using RT and IN transcomplemented HIV-1 viral particles capable of a single round of replication. Our results reveal that the central DNA flap was not essential to HIV-1 replication but conferred a five- to sevenfold infectivity advantage to single-cycle replicating viruses in a variety of cellular systems, including MAGI cells, MT4, dividing and nondividing C8166 T-cell lines, and h-PBMCs (Fig. 1and 2 and data not shown). These results are consistent with findings reported by several previous studies that the central DNA flap conferred a transduction advantage of 2- to 10-fold on vesicular stomatitis virus-G-pseudotyped HIV-1 vectors (11, 13, 17, 29, 30, 33, 34, 37, 45, 46). At this point, it is still unclear why this substantial defect in single-round infectivity caused by disruption of the DNA flap does not translate into a detectable difference when the replication kinetic is monitored by using replication-competent virus, as shown recently (13, 29). Clearly, more studies in this area are required to understand this discrepancy.

In an attempt to understand the mechanism(s) underlying the effect of the central DNA flap during HIV-1 single-cycle replication, we analyzed by PCR the amount of integrated proviral DNA in the presence or absence of the central DNA flap. Our results clearly show that disruption of the DNA flap results in a five- to sevenfold decrease in proviral DNA integration (Fig. 4), suggesting that the central DNA flap contributes to efficient single-cycle viral replication by acting on an early stage(s) of the HIV-1 infection cycle at and/or prior to viral integration. We further analyzed total late-reverse-transcribed DNA products over time during $cPPT^+$ or $cPPT^-$ single-cycle viral infection and determined the distribution of total viral cDNA in the nucleus and the cytoplasm. Our results

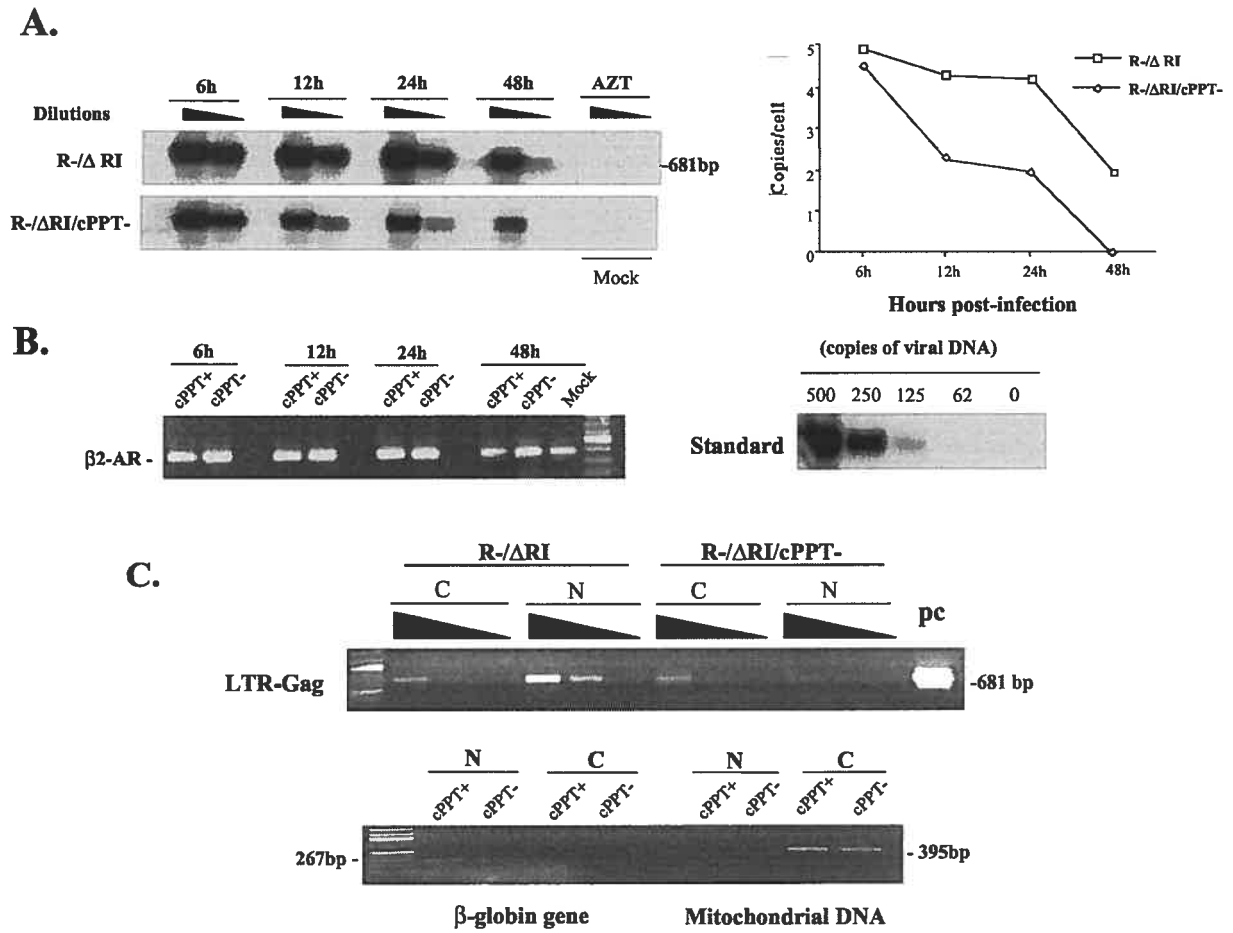


FIG. 4. Effect of the central DNA flap on HIV-1 cDNA nuclear import. (A) h-PBMCs were infected with transcomplemented $R^-/\Delta RI$ ($cPPT^+$) and $R^-/\Delta RI/cPPT^-$ ($cPPT^-$) viruses (125 ng of p24/$10^6$ cells) for 2 h. As the negative control, 3′-azido-3′-deoxythymidine (AZT; 10 μM)-pretreated PBMCs were infected with the same amounts of transcomplemented $R^-/\Delta RI$ virus. At each indicated time point, serial dilutions of extracted total DNA were analyzed for late-reverse-transcription products by PCR by using long terminal repeat (LTR)-Gag primers and Southern blotting. HIV-1 late-reverse-transcription products detected in the left panel were quantified by laser densitometry. The diagram at the right shows the number of HIV-1 cDNA copies per cell as determined by using the PCR-generated standard curve (B, right panel). These results are representative of those obtained in two independent experiments. Serially diluted R-/ΔRI plasmid DNA was used as a standard for DNA copy quantification (right panel). To evaluate cellular DNA levels in each sample, the cellular β2-AR gene was amplified by PCR and visualized by ethidium bromide staining (left panel). (C) At 24-h p.i., $2 \times 10^6$ infected h-PBMCs were fractionated into cytoplasmic and nuclear fractions as described previously (36). The amounts of viral DNA in the cytoplasmic and nuclear fractions were evaluated by PCR by using HIV-1 LTR-Gag primers and were visualized by ethidium bromide staining. The $R^-/\Delta RI$ plasmid DNA was used as a PCR-positive control (pc) (upper panel). In parallel, the purity and DNA content of each subcellular fraction were evaluated by PCR detection of the human globin gene and mitochondrial DNA and were visualized by ethidium bromide staining (lower panel). N, nuclear fraction; C, cytoplasmic fraction.

reveal that the presence of the central DNA flap does not significantly influence the amount of viral transcripts produced at early time points (6 h) but contributes primarily to an accumulation of viral cDNA in the nucleus (Fig. 4). These results are consistent with findings made by several previous studies using either replication-competent viruses or single-round HIV-1 vector transduction systems that the central cDNA flap enhances the establishment of HIV-1 infection by facilitating the nuclear import of proviral DNA (17, 30, 45).

In addition to the effect on viral cDNA nuclear import, our data also suggest that the presence of the central DNA flap might have a stabilizing and/or a protective effect on viral cDNA (Fig. 4). Indeed, it is possible that the central DNA flap might contribute to a correct conformation of viral cDNA and/or be implicated in the recruitment of host cell proteins to form a functional PIC capable of effective proviral DNA nuclear import. Absence of an intact central DNA flap might lead to immature PIC where viral cDNA is less stable or subject to rapid degradation. In this regard, it has recently been reported that the central DNA flap region of viral cDNA was resistant to DNase I digestion when viral PIC complexes were isolated from the cytoplasm of infected cells at 10 h, whereas it was sensitive to degradation when complexes were isolated at 8.5 h after infection (25). Alternatively, it may also be possible that the presence of the central DNA flap positively modulates the stability of viral cDNA in the nucleus. Interestingly, a similar difference in the rate of decline of wt and cPPT-defective total viral cDNA was observed by Limon et al. (29) in infected PBMCs, although the effect was not as pronounced as in our study. Altogether, these observations point toward a possible

role of the central DNA flap in the formation and maturation of HIV-1 PICs: such a role is likely to influence viral cDNA stability and nuclear import.

We thank Nicole Rougeau, Johanne Mercier, and Serge Senechal for technical support. We thank Sylvie Beaulieu, Luchino Y. Cohen, and Ghislaine Duisit for fruitful discussions. We are also grateful to M. Emerman for the HeLa-CD4-β-Gal cells, which were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

Xiaojian Yao is a recipient of a Médecine-Relève 2000-Messenger Foundation Award from the Faculté de Médecine, Université de Montréal. Éric A. Cohen is the recipient of the Canada Research Chair in Human Retrovirology. This work was supported by grants from the Canadian Foundation for AIDS Research (CANFAR) (X.Y), the Canadian Institute of Health Research (CIHR), and the Fonds de la Recherche en Santé du Québec (FRSQ) (E.A.C.).

## REFERENCES

1. **Ansari-Lari, M. A., L. A. Donehower, and R. A. Gibbs.** 1995. Analysis of human immunodeficiency virus type 1 integrase mutants. Virology **211**:332–335.
2. **Ansari-Lari, M. A., and R. A. Gibbs.** 1996. Expression of human immunodeficiency virus type 1 reverse transcriptase in *trans* during virion release and after infection. J. Virol. **70**:3870–3876.
3. **Bouyac-Bertoia, M., J. D. Dvorin, R. A. Fouchier, Y. Jenkins, B. E. Meyer, L. I. Wu, M. Emerman, and M. H. Malim.** 2001. HIV-1 infection requires a functional integrase NLS. Mol. Cell **7**:1025–1035.
4. **Bukovsky, A., and H. Gottlinger.** 1996. Lack of integrase can markedly affect human immunodeficiency virus type 1 particle production in the presence of an active viral protease. J. Virol. **70**:6820–6825.
5. **Bukrinsky, M., and O. K. Haffar.** 1998. HIV-1 nuclear import: matrix protein is back on center stage, this time together with Vpr. Mol. Med. **4**:138–143.
6. **Bukrinsky, M. I., S. Haggerty, M. P. Dempsey, N. Sharova, A. Adzhubel, L. Spitz, P. Lewis, D. Goldfarb, M. Emerman, and M. Stevenson.** 1993. A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. Nature **365**:666–669.
7. **Charneau, P., M. Alizon, and F. Clavel.** 1992. A second origin of plus-strand synthesis is required for optimal human immunodeficiency virus replication. J. Virol. **66**:2814–2820.
8. **Charneau, P., G. Mirambeau, P. Roux, S. Paulous, H. Buc, and F. Clavel.** 1994. HIV-1 reverse transcription: a termination step at the center of the genome. J. Mol. Biol. **241**:651–662.
9. **Chun, T. W., L. Stuyver, S. B. Mizell, L. A. Ehler, J. A. Mican, M. Baseler, A. L. Lloyd, M. A. Nowak, and A. S. Fauci.** 1997. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc. Natl. Acad. Sci. USA **94**:13193–13197.
10. **Coffin, J. M.** 1990. *Retroviridae* and their replication. *In* B. N. Fields and D. M. Knipe (ed.), Fields virology, 2nd ed. Raven Press, New York, N.Y., p. 1437–1500.
11. **Dardalhon, V., B. Herpers, N. Noraz, F. Pflumio, D. Guetard, C. Leveau, A. Dubart-Kupperschmitt, P. Charneau, and N. Taylor.** 2001. Lentivirus-mediated gene transfer in primary T cells is enhanced by a central DNA flap. Gene Ther. **8**:190–198.
12. **de Noronha, C. M., M. P. Sherman, H. W. Lin, M. V. Cavrois, R. D. Moir, R. D. Goldman, and W. C. Greene.** 2001. Dynamic disruptions in nuclear envelope architecture and integrity induced by HIV-1 Vpr. Science **294**:1105–1108.
13. **Dvorin, J. D., P. Bell, G. G. Maul, M. Yamashita, M. Emerman, and M. H. Malim.** 2002. Reassessment of the roles of integrase and the central DNA flap in human immunodeficiency virus type 1 nuclear import. J. Virol. **76**:12087–12096.
14. **Engelman, A., G. Englund, J. M. Orenstein, M. A. Martin, and R. Craigie.** 1995. Multiple effects of mutations in human immunodeficiency virus type 1 integrase on viral replication. J. Virol. **69**:2729–2736.
15. **Farnet, C. M., and W. A. Haseltine.** 1996. HIV cDNA integration: molecular biology and inhibitor development. AIDS **10**:S3–S11.
16. **Fletcher, T. M., III, M. A. Soares, S. McPhearson, II. Hui, M. Wiskerchen, M. A. Muesing, G. M. Shaw, A. D. Leavitt, J. D. Boeke, and B. H. Hahn.** 1997. Complementation of integrase function in HIV-1 virions. EMBO J. **16**:5123–5138.
17. **Follenzi, A., L. E. Ailles, S. Bakovic, M. Geuna, and L. Naldini.** 2000. Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 *pol* sequences. Nat. Genet. **25**:217–222.
18. **Forget, J., X. J. Yao, J. Mercier, and E. A. Cohen.** 1998. Human immunodeficiency virus type 1 Vpr protein transactivation function: mechanism and identification of domains involved. J. Mol. Biol. **284**:915–923.
19. **Fouchier, R. A., B. E. Meyer, J. H. Simon, U. Fischer, A. V. Albright, F. Gonzalez-Scarano, and M. H. Malim.** 1998. Interaction of the human immunodeficiency virus type 1 Vpr protein with the nuclear pore complex. J. Virol. **72**:6004–6013.
20. **Gallay, P., T. Hope, D. Chin, and D. Trono.** 1997. HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. Proc. Natl. Acad. Sci. USA **94**:9825–9830.
21. **Goff, S. P.** 1992. Genetics of retroviral integration. Annu. Rev. Genet. **26**:527–544.
22. **Greene, W. C., and B. M. Peterlin.** 2002. Charting HIV's remarkable voyage through the cell: basic science as a passport to future therapy. Nat. Med. **8**:673–680.
23. **Haffar, O. K., S. Popov, L. Dubrovsky, I. Agostini, H. Tang, T. Pushkarsky, S. G. Nadler, and M. Bukrinsky.** 2000. Two nuclear localization signals in the HIV-1 matrix protein regulate nuclear import of the HIV-1 pre-integration complex. J. Mol. Biol. **299**:359–368.
24. **Heinzinger, N. K., M. I. Bukinsky, S. A. Haggerty, A. M. Ragland, V. Kewalramani, M. A. Lee, H. E. Gendelman, L. Ratner, M. Stevenson, and M. Emerman.** 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc. Natl. Acad. Sci. USA **91**:7311–7315.
25. **Khiytani, D. K., and N. J. Dimmock.** 2002. Characterization of a human immunodeficiency virus type 1 pre-integration complex in which the majority of the cDNA is resistant to DNase I digestion. J. Gen. Virol. **83**:2523–2532.
26. **Kimpton, J., and M. Emerman.** 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated β-galactosidase gene. J. Virol. **66**:2232–2239.
27. **Le Rouzic, E., A. Mousnier, C. Rustum, F. Stutz, E. Hallberg, C. Dargemont, and S. Benichou.** 2002. Docking of HIV-1 Vpr to the nuclear envelope is mediated by the interaction with the nucleoporin hCG1. J. Biol. Chem. **277**:45091–45098.
28. **Lewis, P. F., and M. Emerman.** 1994. Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. J. Virol. **68**:510–516.
29. **Limon, A., N. Nakajima, R. Lu, H. Z. Ghory, and A. Engelman.** 2002. Wild-type levels of nuclear localization and human immunodeficiency virus type 1 replication in the absence of the central DNA flap. J. Virol. **76**:12078–12086.
30. **Maele, B. V., J. D. Rijck, E. D. Claercq, and Z. Debyser.** 2003. Impact of the central polypurine tract on the kinetics of human immunodeficiency virus type 1 vector transduction. J. Virol. **77**:4685–4694.
31. **Maertens, G., P. Cherepanov, W. Pluymers, K. Busschots, E. De Clercq, Z. Debyser, and Y. Engelborghs.** 2003. LEDGF p75 is essential for nuclear and chromosomal targeting of HIV-1 integrase in human cells. J. Biol. Chem. **278**:33528–33539.
32. **Nie, Z., D. Bergeron, R. A. Subbramanian, X. J. Yao, F. Checroune, N. Rougeau, and E. A. Cohen.** 1998. The putative alpha helix 2 of human immunodeficiency virus type 1 Vpr contains a determinant which is responsible for the nuclear translocation of proviral DNA in growth-arrested cells. J. Virol. **72**:4104–4115.
33. **Park, F., and M. A. Kay.** 2001. Modified HIV-1 based lentiviral vectors have an effect on viral transduction efficiency and gene expression in vitro and in vivo. Mol. Ther. **4**:164–173.
34. **Parolin, C., B. Taddeo, G. Palu, and J. Sodroski.** 1996. Use of *cis*- and *trans*-acting viral regulatory sequences to improve expression of human immunodeficiency virus vectors in human lymphocytes. Virology **222**:415–422.
35. **Petit, C., O. Schwartz, and F. Mammano.** 2000. The karyophilic properties of human immunodeficiency virus type 1 integrase are not required for nuclear import of proviral DNA. J. Virol. **74**:7119–7126.
36. **Simon, J. H. M., and M. H. Malim.** 1996. The human immunodeficiency virus type 1 Vif protein modulates the postpenetration stability of viral nucleoprotein complexes. J. Virol. **70**:5297–5305.
37. **Sirven, A., F. Pflumio, V. Zennou, M. Titeux, W. Vainchenker, L. Coulombel, A. Dubart-Kupperschmitt, and P. Charneau.** 2000. The human immunodeficiency virus type-1 central DNA flap is a crucial determinant for lentiviral vector nuclear import and gene transduction of human hematopoietic stem cells. Blood **96**:4103–4110.
38. **Vandegraaff, N., R. Kumar, H. Hocking, T. R. Burke, Jr., J. Mills, D. Rhodes, C. J. Burrell, and P. Li.** 2001. Specific inhibition of human immunodeficiency virus type 1 (HIV-1) integration in cell culture: putative inhibitors of HIV-1 integrase. Antimicrob. Agents Chemother. **45**:2510–2516.
39. **Vodicka, M. A., D. M. Koepp, P. A. Silver, and M. Emerman.** 1998. HIV-1 Vpr interacts with the nuclear transport pathway to promote macrophage infection. Genes Dev. **12**:175–185.
40. **Weinberg, J. B., T. J. Matthews, B. R. Cullen, and M. H. Malim.** 1991. Productive human immunodeficiency virus type 1 (HIV-1) infection of nonproliferating human monocytes. J. Exp. Med. **174**:1477–1482.
41. **Wu, X., H. Liu, H. Xiao, J. A. Conway, E. Hunter, and J. C. Kappes.** 1997. Functional RT and IN incorporated into HIV-1 particles independently of the Gag/Pol precursor protein. EMBO J. **16**:5113–5122.
42. **Yao, X. J., G. Kobinger, S. Dandache, N. Rougeau, and E. Cohen.** 1999. HIV-1 Vpr-chloramphenicol acetyltransferase fusion proteins: sequence re-

quirement for virion incorporation and analysis of antiviral effect. Gene Ther. 6:1590–1599.
43. **Yao, X. J., A. J. Mouland, R. A. Subbramanian, J. Forget, N. Rougeau, D. Bergeron, and E. A. Cohen.** 1998. Vpr stimulates viral expression and induces cell killing in human immunodeficiency virus type 1-infected dividing Jurkat T cells. J. Virol. 72:4686–4693.
44. **Yao, X. J., R. A. Subbramanian, N. Rougeau, F. Boisvert, D. Bergeron, and E. A. Cohen.** 1995. Mutagenic analysis of human immunodeficiency virus type 1 Vpr: role of a predicted N-terminal alpha-helical structure in Vpr nuclear localization and virion incorporation. J. Virol. 69:7032–7044.
45. **Zennou, V., C. Petit, D. Guetard, U. Nerhbass, L. Montagnier, and P. Charneau.** 2000. HIV-1 genome nuclear import is mediated by a central DNA flap. Cell 101:173–185.
46. **Zennou, V., C. Serguera, C. Sarkis, P. Colin, E. Perret, J. Mallet, and P. Charneau.** 2001. The HIV-1 DNA flap stimulates HIV vector-mediated cell transduction in the brain. Nat. Biotechnol. 19:446–450.

APPENDIX II

Retrovirology

BioMed Central

Research

Open Access

# Contribution of the C-terminal tri-lysine regions of human immunodeficiency virus type 1 integrase for efficient reverse transcription and viral DNA nuclear import

Zhujun Ao[1,2,3], Keith R Fowke[2], Éric A Cohen[3] and Xiaojian Yao*[1,2,3]

Address: [1]Laboratory of Molecular Human Retrovirology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba R3E 0W3, Canada, [2]Department of Medical Microbiology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba R3E 0W3, Canada and [3]Laboratory of Human Retrovirology, Institut de Recherches Cliniques de Montréal, Département de microbiologie et immunologie, Faculté de Médecine, Université de Montréal, Montréal, Quebec H2W 1R7, Canada

Email: Zhujun Ao - [redacted] Keith R Fowke - [redacted] Éric A Cohen - [redacted] Xiaojian Yao* - [redacted]

* Corresponding author

Published: 18 October 2005

*Retrovirology* 2005, 2:62 doi:10.1186/1742-4690-2-62

Received: 05 August 2005
Accepted: 18 October 2005

This article is available from: http://www.retrovirology.com/content/2/1/62



## Abstract

**Background:** In addition to mediating the integration process, HIV-1 integrase (IN) has also been implicated in different steps during viral life cycle including reverse transcription and viral DNA nuclear import. Although the karyophilic property of HIV-1 IN has been well demonstrated using a variety of experimental approaches, the definition of domain(s) and/or motif(s) within the protein that mediate viral DNA nuclear import and its mechanism are still disputed and controversial. In this study, we performed mutagenic analyses to investigate the contribution of different regions in the C-terminal domain of HIV-1 IN to protein nuclear localization as well as their effects on virus infection.

**Results:** Our analysis showed that replacing lysine residues in two highly conserved tri-lysine regions, which are located within previously described Region C ($^{235}$WKGPAKLLWKGEGAVV) and sequence Q ($^{211}$KELQKQITK) in the C-terminal domain of HIV-1 IN, impaired protein nuclear accumulation, while mutations for $RK_{263,4}$ had no significant effect. Analysis of their effects on viral infection in a VSV-G pseudotyped RT/IN trans-complemented HIV-1 single cycle replication system revealed that all three C-terminal mutant viruses (KK215,9AA, KK240,4AE and RK263,4AA) exhibited more severe defect of induction of β-Gal positive cells and luciferase activity than an IN class I mutant D64E in HeLa-CD4-CCR5-β-Gal cells, and in dividing as well as non-dividing C8166 T cells, suggesting that some viral defects are occurring prior to viral integration. Furthermore, by analyzing viral DNA synthesis and the nucleus-associated viral DNA level, the results clearly showed that, although all three C-terminal mutants inhibited viral reverse transcription to different extents, the KK240,4AE mutant exhibited most profound effect on this step, whereas KK215,9AA significantly impaired viral DNA nuclear import. In addition, our analysis could not detect viral DNA integration in each C-terminal mutant infection, even though they displayed various low levels of nucleus-associated viral DNA, suggesting that these C-terminal mutants also impaired viral DNA integration ability.

**Conclusion:** All of these results indicate that, in addition to being involved in HIV-1 reverse transcription and integration, the C-terminal tri-lysine regions of IN also contribute to efficient viral DNA nuclear import during the early stage of HIV-1 replication.

## Background

The integrase (IN) of human immunodeficiency virus type 1 (HIV-1) is encoded by the *pol* gene and catalyzes integration of viral cDNA into host chromosome, an essential step in HIV-1 replication. In addition to mediating the integration process, HIV-1 IN also participates in different steps during viral life cycle, including reverse transcription and viral DNA nuclear import [1-6]. During early phase of the HIV-1 replication cycle, after virus entry into target cells, another *pol* gene product, reverse transcriptase (RT), copies viral genomic RNA into double-stranded cDNA which exists within a nucleoprotein pre-integration complex (PIC). The PIC also contains viral proteins including RT, IN, nucleocapsid (NC, p9), Vpr and matrix (MA, p17) and this large nucleoprotein complex is capable of actively translocating into the cell nucleus, including that of non-dividing cells (reviewed in reference [7]). This feature is particularly important for the establishment of HIV-1 replication and pathogenesis in exposed hosts, since the infection of postmitotic cells including tissue macrophages, mucosal dendritic cells as well as non-dividing T cells may be essential not only for viral transmission and dissemination, but also for the establishment of persistent viral reservoirs.

HIV-1 IN is composed of three functional domains, an N-terminal domain, a central catalytic core domain and a C-terminal domain, all of which are required for a complete integration reaction. The N-terminal domain harbors an HHCC-type zinc binding domain and is implicated in the multimerization of the protein and contributes to the specific recognition of DNA ends [8-10]. The core domain of IN contains the highly conserved DDE motif which is important for catalytic activity of the protein [11,12]. The C-terminal domain was shown to possess nonspecific DNA binding properties [13,14]. Some mutations within this region cause a drastic loss of virus infectivity without affecting the enzymatic activity of IN *in vitro* [2,13-16]. There are three conserved sequences in the C-terminus of IN that are essential for HIV-1 replication. Regions C ($^{235}$WKGPAKLLWKGEGAVV) and N ($^{259}$VVPRRKAK) are conserved in all known retroviruses and the $^{211}$KELQKQITK motif falls within the so-called glutamine-rich based region (sequence Q) of lentiviruses [17]. Alteration of each of the three sequences such as Q214L/Q216L, K215A/K219A, W235E, K236A/K240A, K244A/E246A, RRE263-5AAH resulted in loss of viral replication [15-18]. However, the mechanism(s) underlying the loss of viral infectivity remains controversial.

A number of studies have demonstrated the karyophilic properties of IN implicating that this protein may play an important role for PIC nuclear import [3,19-23]. However, the definition of nuclear localization signals (NLSs) in IN as well as their contribution to HIV-1 PIC nuclear import still remain to be determined. Previous report has suggested an atypical bipartite NLS ($^{186}$KRK and $^{211}$KELQKQITK) by showing that IN mutants K186Q and Q214/216L in these regions lost the protein nuclear localization and their inability to bind to karyopherin α *in vitro* [3]. However, in attempt to analyze the effect of these mutants during HIV-1 replication, other studies did not reveal the importance of these IN mutants (K186Q and Q214/216L) for viral nuclear import; rather they appear to be required for reverse transcription, integration or undefined post-nuclear entry steps [16,18,23]. Also, another IN amino acid sequence IIGQVRDQAEHLK (aa161–173), was initially identified as an atypical NLS, which is required for viral DNA nuclear import [19]. However, reassessments of this putative NLS function failed to confirm this conclusion [24,25]. Some reports have also acknowledged that IN localization could result from passive diffusion of the protein and its DNA binding property [26,27], but DNA binding alone does not fully explain a rapid, ATP- and temperature-dependent nuclear import of IN [20]. It has recently been reported that the nuclear translocation of HIV-1 IN can be attributed to its interaction with a cellular component, human lens epithelium-derived growth factor/transcription coactivator p75 (LEDGF/p75) and LEDGF/p75 was also shown to be a component of HIV PIC [28,29]. However, whether this IN/LEDGF/p75 interaction plays an important role for HIV-1 nuclear import still remains to be elucidated, since HIV-1 infection and replication in LEDGF/p75-deficient cells was equivalent to that in control cells, regardless whether cells were dividing or growth arrested [29]. Thus, even though extensive studies have been dedicated in this specific research field, the contribution of HIV-1 IN to viral PIC nuclear import remains to be defined.

In this study, we have performed substitution mutational analysis to investigate the contribution of different C-terminal regions of IN to protein nuclear localization and their effects on HIV-1 replication. Our results showed that mutations of lysine residues in two tri-lysine regions, which are located within previously described Region C and sequence Q [17] in the C-terminal domain of HIV-1 IN, impaired protein nuclear localization, while mutations of arginines at amino acid position of 263 and 264 in the distal part of the C-terminal domain of IN had no significant effect. Moreover, we assessed the effect of these IN mutants during HIV-1 single cycle infection mediated by VSV-G pseudotyped RT/IN trans-complemented viruses. Results showed that, while all three C-terminal mutant viruses differentially affected HIV-1 reverse transcription, the KK240,4AE mutant exhibited most profound inhibition on this step, whereas KK215,9AA significantly impaired viral DNA nuclear import.

## Results

### *The C-terminal domain of HIV-1 integrase (IN) is required for the nuclear localization of IN-YFP fusion protein*

In this study, we first investigated the intracellular localization of HIV-1 IN and delineated the region(s) of IN contributing to its karyophilic property. A HIV-1 IN-YFP fusion protein expressor (CMV-IN-YFP) was generated by fusing a full-length HIV-1 IN cDNA (amplified from HIV-1 HxBru molecular clone [30]) to the 5' end of YFP cDNA in a CMV-IN-YFP expressor, as described in Materials and Methods. Transfection of CMV-IN-YFP expressor in 293T cells resulted in the expression of a 57 kDa IN-YFP fusion protein (Fig. 1B, lane 2; Fig. 2B, lane 1), whereas expression of YFP alone resulted in a 27 kDa protein (Fig. 2B, lane 5). Given that HeLa cells have well-defined morphology and are suitable for observation of intracellular protein distribution, we tested the intracellular localization of YFP and IN-YFP by transfecting CMV-IN-YFP or CMV-YFP expressor in HeLa cells. After 48 hours of transfection, cells were fixed and subjected to indirect immunofluorescence assay using primary rabbit anti-GFP antibody followed by secondary FITC-conjugated anti-rabbit antibodies. Results showed that, in contrast to a diffused intracellular localization pattern of YFP (data not shown), the IN-YFP fusion protein was predominantly localized in the nucleus (Fig 1C, a1), confirming the karyophilic feature of HIV-1 IN.

To delineate the karyophilic determinant in HIV-1 IN, two truncated IN-YFP expressors CMV-$IN_{50-288}$-YFP and CMV-$IN_{1-212}$-YFP were generated. In CMV-$IN_{50-288}$-YFP, the N-terminal HH-CC domain of IN (aa 1–49) was deleted and in CMV-$IN_{1-212}$-YFP, the C-terminal domain (aa 213–288) was removed (Fig. 1A). Transfection of each truncated IN-YFP fusion protein expressor in 293T cells resulted in the expression of $IN_{50-288}$-YFP and $IN_{1-212}$-YFP at approximately 52 kDa and 48 kDa molecular mass respectively (Fig. 1B, lanes 3 and 4). We next investigated the intracellular localization of truncated IN-YFP fusion proteins in HeLa cells by using indirect immunofluorescence assay, as described above. Results showed that the $IN_{50-288}$-YFP was predominantly localized in the nucleus with a similar pattern as the wild-type IN-YFP fusion protein (Fig. 1C, compare b1 to a1). However, $IN_{1-212}$-YFP fusion protein was excluded from the nucleus, with an accumulation of the mutant protein in the cytoplasm (Fig 1C, c1). These results were also further confirmed by using rabbit anti-IN antibody immunofluorescence assay (data not shown). Taken together, our data show that the C-terminal domain of HIV-1 IN is required for its nuclear accumulation.

### *Two tri-lysine regions in the C-terminal domain of IN are involved in the protein nuclear localization*

The C-terminal domain of HIV-1 IN contains several regions that are highly conserved in different HIV-1 strains, including Q, C and N regions [17]. Interestingly, in regions Q and C, sequences of $^{211}$KELQKQITK and $^{236}$KGPAKLLWK possess high similarity in terms of numbers and position of lysine residues and therefore, we term them proximal tri-lysine region and distal tri-lysine region, respectively (Fig. 2A). All of these lysine residues are highly conserved in most HIV-1 strains [31]. To test whether these basic lysine residues could constitute for a possible nuclear localization signal for IN nuclear localization, we specifically introduced substitution mutations for two lysines in each tri-lysine region and generated $IN_{KK215,9AA}$-YFP and $IN_{KK240,4AE}$-YFP expressors (Fig. 2A). In the conserved N region, there is a stretch of four basic residues among five amino acids (aa) $^{262}$RRKAK. To characterize whether this basic aa region may contributes to IN nuclear localization, we replaced an arginine and a lysine at positions of 263 and 264 by alanines in this region and generated a mutant ($IN_{RK263,4AA}$-YFP). The protein expression of different IN-YFP mutants in 293T cells showed that, like the wild type IN-YFP, each IN-YFP mutant fusion protein was detected at similar molecular mass (57 kDa) in SDS-PAGE (Fig 2B, lanes 1 to 4), while YFP alone was detected at position of 27 kDa (lane 5). Then, the intracellular localization of each IN mutant was investigated in HeLa cells by using similar methods, as described above. Results showed that, while the wild type IN-YFP and $IN_{RK263,4AA}$-YFP still predominantly localized to the nucleus (Fig. 2C, a1 and d1), both $IN_{KK215,9AA}$-YFP and $IN_{KK240,4AE}$-YFP fusion proteins were shown to distribute throughout the cytoplasm and nucleus, but with much less intensity in the nucleus (Fig 2C, a1 and b1). These data suggest that these lysine residues in each tri-lysine regions are required for efficient HIV-1 IN nuclear localization.

### *Production of VSV-G pseudotyped HIV-1 IN mutant viruses and their effects on HIV-1 infection*

Given that two di-lysine mutants located in the C-terminal domain of IN are involved in HIV-1 IN nuclear localization, we next evaluated whether these IN mutants would affect the efficiency of HIV-1 infection. To specifically analyze the effect of IN mutants in early steps of viral infection, we modified a previously described HIV-1 single-cycle replication system [32] and constructed a RT/IN/Env gene-deleted HIV-1 provirus NLlucΔBglΔRI, in which the *nef* gene was replaced by a firefly luciferase gene [33]. Co-expression of NLlucΔBglΔRI provirus with Vpr-RT-IN expressor and a vesicular stomatitis virus G (VSV-G) glycoprotein expressor will produce viral particles that can undergo a single-round of replication, since RT, IN and Env defects of provirus will be complemented *in trans* by

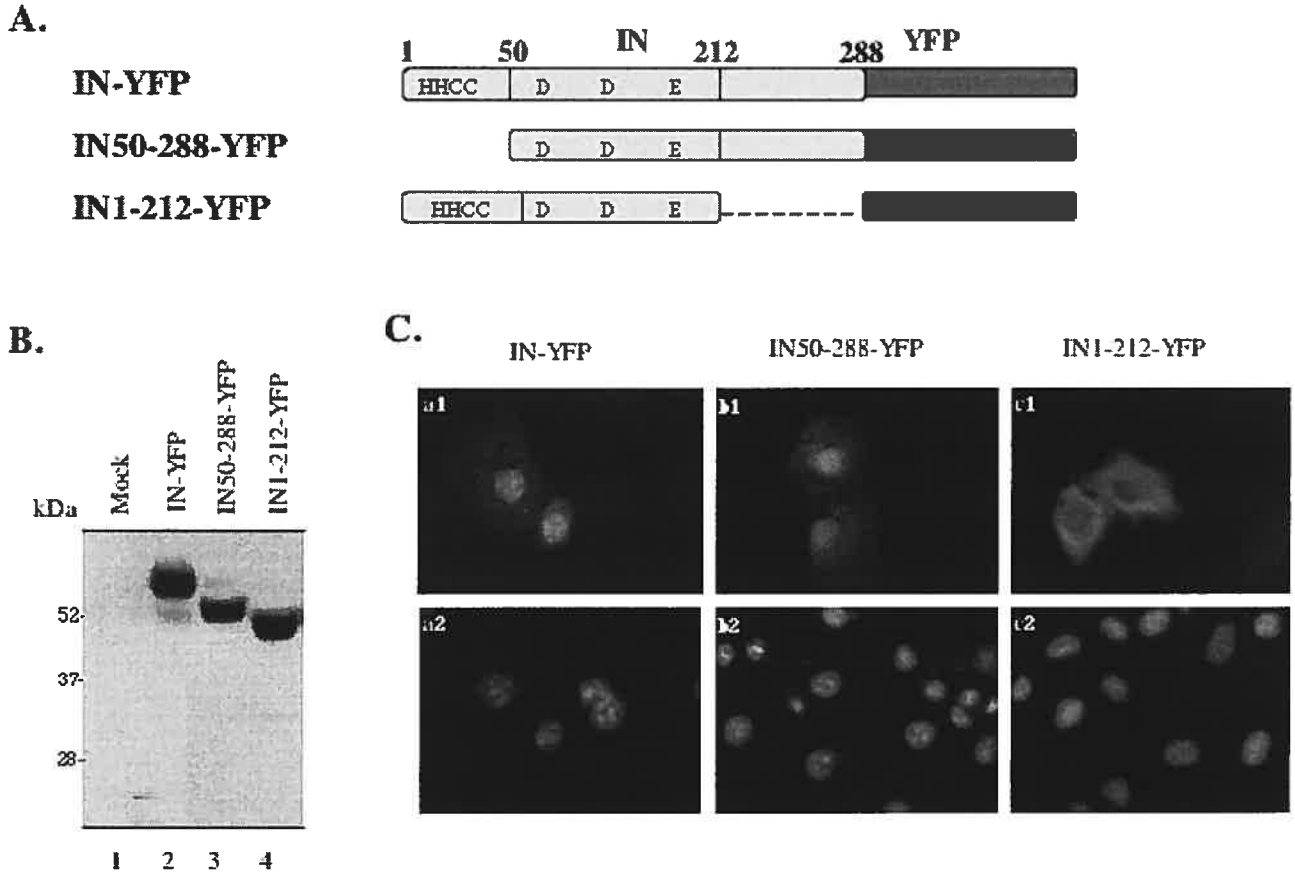


**Figure 1**

**Subcellular localization of the wild-type and truncated HIV integrase fused with YFP**. A) Schematic structure of HIV-1 integrase-YFP fusion proteins. Full-length (1–288aa) HIV-1 integrase, the N-terminus-truncated mutant (51–228aa) or the C-terminus-truncated mutant (1–212aa) was fused in frame at the N-terminus of YFP protein. The cDNA encoding for each IN-YFP fusion protein was inserted in a SVCMV expression plasmid. B) Expression of different IN-YFP fusion proteins in 293T cells. 293T cells were transfected with each IN-YFP expressor and at 48 hours of transfection, cells were lysed, immunoprecipitated with anti-HIV serum and resolved by electrophoresis through a 12.5% SDS-PAGE followed by Western blot with rabbit anti-GFP antibody. The molecular weight markers are indicated at the left side of the gel. C) Intracellular localization of different IN-YFP fusion proteins. HeLa cells were transfected with each HIV-1 IN-YFP fusion protein expressor and at 48 hours of transfection, cells were fixed and subjected to indirect immunofluorescence using rabbit anti-GFP and then incubated with FITC-conjugated anti-rabbit antibodies. The localization of each fusion protein was viewed by Fluorescence microscopy with a 50× oil immersion objective. Upper panel is fluorescence images and bottom panel is DAPI nucleus staining.

VSV-G glycoprotein and Vpr-mediated RT and IN trans-incorporation [32]. This single cycle replication system allows us to introduce different mutations into IN gene sequence without differentially affecting viral morphogenesis and the activity of the central DNA Flap. After different IN mutations KK215,9AA, KK240,4AE and RR263,4AA were introduced into Vpr-RT-IN expressor, we produced VSV-G pseudotyped HIV-1 IN mutant virus stocks in 293T cells. In order to specifically investigate the effect of IN mutants on early steps during HIV-1 infection prior to integration, an IN class I mutant D64E was also included as control. After each viral stock was produced (as indicated in Fig. 3A), similar amounts of each virus stock (quantified by virion-associated RT activity) were lysed and virus composition and trans-incorporation of RT and IN of each virus stock were analyzed by Western blot analysis with anti-IN and anti-HIV antibodies, as described in Materials and Methods. Results showed that

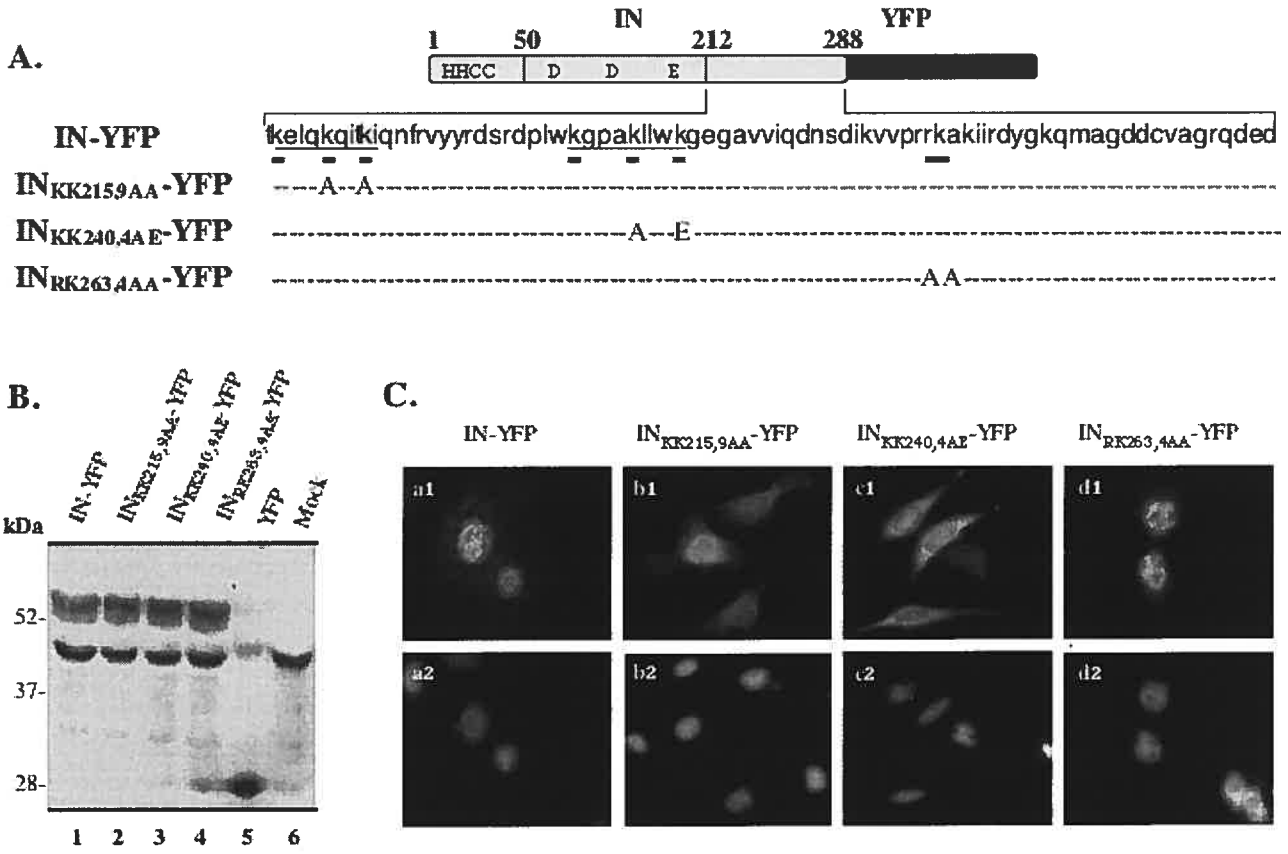


**Figure 2**

**Effect of different IN C-terminal substitution mutants on IN-YFP intracellular localization.** A) Diagram of HIV-1 IN domain structure and introduced mutations at the C-terminal domain of the protein. The position of lysines in two tri-lysine regions and introduced mutations are shown at the bottom of sequence. B) The expression of the wild-type and mutant IN-YFP fusion proteins were detected in transfected 293T cells by using immunoprecipitation with anti-HIV serum and Western blot with rabbit anti-GFP antibody, as described in figure 1. The molecular weight markers are indicated at the left side of the gel. C) Intracellular localization of different HIV-1 IN mutant-YFP fusion proteins in HeLa cells were analyzed by fluorescence microscopy with a 50× oil immersion objective. The nucleus of HeLa cells was simultaneously visualized by DAPI staining (lower panel).

all VSV-G pseudotyped IN mutant viruses had similar levels of Gagp24, IN and RT, as compared to the wild-type virus (Fig. 3A), indicating that trans-incorporation of RT and IN as well as HIV-1 Gag processing were not differentially affected by the introduced IN mutations.

To test the infectivity of different IN mutant viruses in HeLa-CD4-CCR5-LTR-β-Gal cells, we first compared the infectivity of VSV-G pseudotyped wild type virus and the D64E mutant virus. At 48 hours post-infection with equivalent amount of each virus stock (at 1 cpm RT activity/cell), the number of β-Gal positive cells was evaluated by MAGI assay, as described previously [34]. Results showed that the number of infected cells (β-Gal positive cells) for D64E mutant reached approximately 14% of the wild type level (data not shown). This result is consistent with a previous report showing that, in HeLa MAGI assay, the infectivity level of class I IN integration-defect mutant was approximately 20 to 22% of wild type level [15]. It indicates that, even though the IN mutant D64E virus is defective for integrating viral DNA into host genome, *tat* expression from nucleus-associated and unintegrated viral DNAs can activate HIV-1 LTR-driven β-Gal expression in HeLa-CD4-CCR5-LTR-β-Gal cells. Indeed, several studies have already shown that HIV infection leads to selective transcription of *tat* and *nef* genes before integration

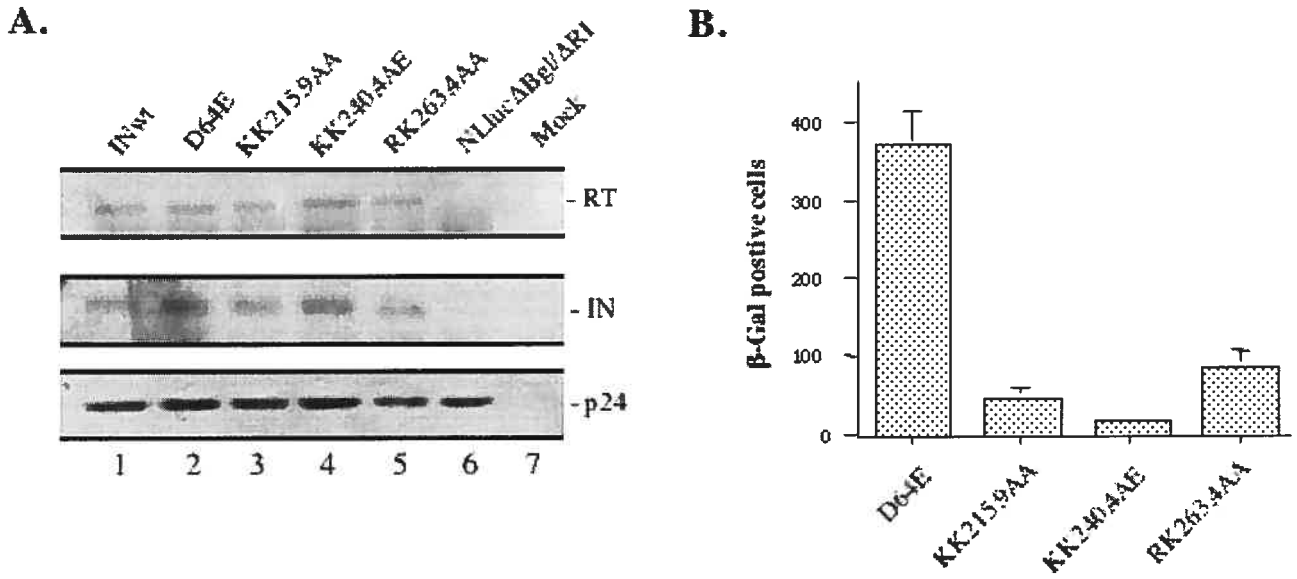


**Figure 3**

**Production of different single-cycle replicating viruses and their infection in HeLa-CD4-CCR5-β-Gal cells.** A). To evaluate the trans-incorporation of RT and IN in VSV-G pseudotyped viral particles, viruses released from 293T cells transfected with NLlucΔBglΔRI provirus alone (lane 6) or cotransfected with different Vpr-RT-IN expressors and a VSV-G expressor (lane 1 to 5) were lysed, immunoprecipitated with anti-HIV serum. Then, immunoprecipitates were run in 12% SDS-PAGE and analyzed by Western blot with rabbit anti-IN antibody (middle panel) or anti-RT and anti-p24 monoclonal antibody (upper and lower panel). B) The infectivity of trans-complemented viruses produced in 293 T cells was evaluated by MAGI assay. HeLa-CD4-CCR5-LTR-β-Gal cells were infected with equal amounts (at 10 cpm/cell) of different IN mutant viruses and after 48 hours of infection, numbers of β-Gal positive cells (infected cell) were monitored by X-gal staining. Error bars represent variation between duplicate samples and the data is representative of results obtained in three independent experiments.

[2,35,36]. Therefore, this HeLa-CD4-CCR5-LTR-β-Gal cell infection system provides an ideal method for us to evaluate the effect of different IN mutants on early steps of viral infection prior to integration. We next infected HeLa-CD4-CCR5-LTR-β-Gal cells with different VSV-G pseudotyped IN mutant viruses at higher infection dose of 10 cpm RT activity/cell and numbers of β-Gal positive cells were evaluated by MAGI assay after 48 hours of infection. Interestingly, results showed that the IN mutant D64E virus infection induced the highest level of β-Gal positive cells, whereas infection with viruses containing IN mutants KK215,9AA, KK240,4AE or RK263,4AA yielded much lower levels of β-Gal positive cells, which only reached approximately 11%, 5% or 26% of the level of D64E virus infection (Fig. 3B). Based on these results, we reasoned that these IN C-terminal mutants blocked infection mostly by affecting earlier steps of HIV-1 life cycle, such as reverse transcription and/or viral DNA nuclear import steps, which are different from the action of D64E mutant on viral DNA integration.

### *Effect of IN mutants on viral infection in dividing and non-dividing C8166 T cells*

To further test whether these C-terminal mutants could induce similar phenotypes in CD4+ T cells, we infected dividing and non-dividing (aphidicolin-treated) C8166 CD4+ T cells with equal amounts of VSV-G pseudotyped IN mutant viruses (at 5 cpm of RT activity/cell). Since all IN mutant viruses contain a luciferase (luc) gene in place of the *nef* gene, viral infection can be monitored by using a sensitive luc assay which could efficiently detect viral gene expression from integrated and unintegrated viral DNA [33]. After 48 hours of infection, equal amounts of cells were lysed in 50 μl of luc lysis buffer and then, 10 μl of cell lysates was used for measurement of luc activity, as described in Materials and Methods. Results showed that the D64E mutant infection in dividing C8166 T cells induced 14.3 × $10^4$ RLU of luc activity (Fig. 4A), which was approximately 1000-fold lower than that in the wild type virus infection (data not shown). This level of luc activity detected in D64E mutant infection is mostly due

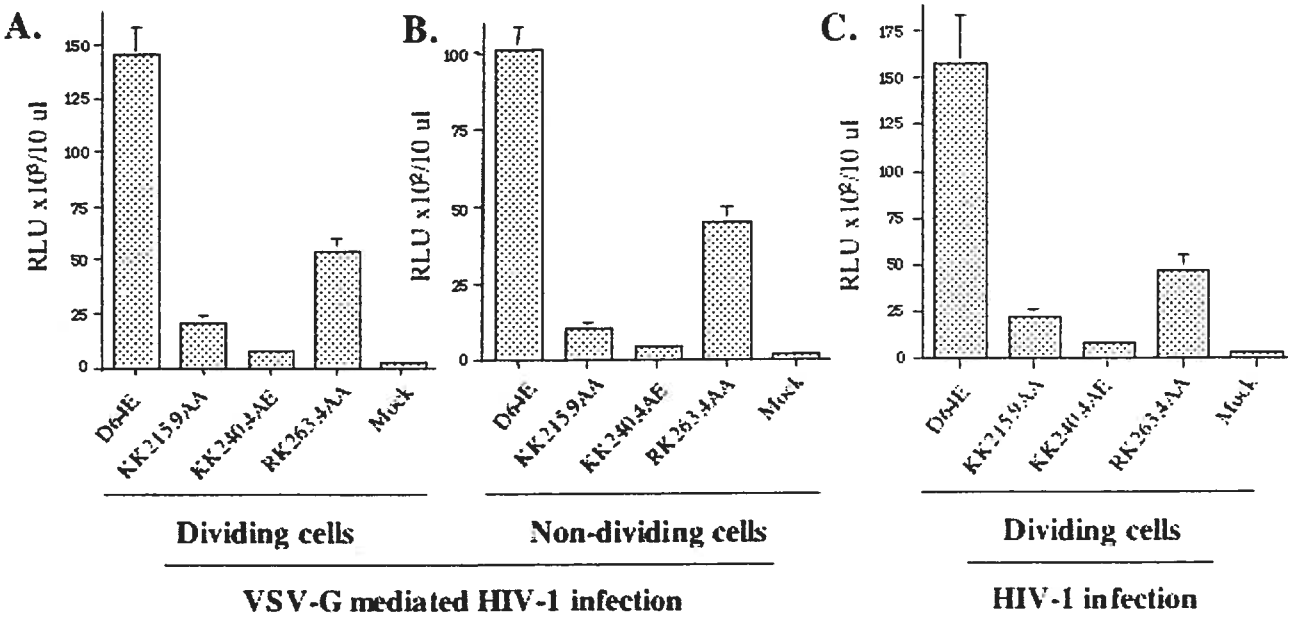


**Figure 4**

**Effect of IN mutants on viral infection in dividing and nondividing C8166 T cells.** To test the effect of different IN mutants on HIV-1 infection in CD4+ T cells, dividing (panel A) and non-dividing (aphidicolin-treated, panel B) C8166 T cells were infected with equal amount of VSV-G pseudotyped IN mutant viruses (at 5 cpm/cell). For evaluation of the effect of different IN mutants on HIV-1 envelope-mediated infection in CD4+ T cells, dividing C8166 T cells were infected with equal amount of HIV-1 envelope competent IN mutant viruses (at 10 cpm/cell) (panel C). After 48 hours of infection, HIV-1 DNA-mediated luciferase induction was monitored by luciferase assay. Briefly, the same amount ($10^6$ cells) of cells was lysed in 50 ul of luciferase lysis buffer and then, 10 μl of cell lysate was subjected to the luciferase assay. Error bars represent variation between duplicate samples and the data is representative of results obtained in three independent experiments.

to *nef* gene expression from the unintegrated DNA [33]. In agreement with the finding by MAGI assay described in figure 3, the Luc activity detected in KK215,9AA, KK240,4AE and RK263,4AA mutant samples were approximately 13%, 5% and 36% of level of D64E mutant infection (Fig. 4A). In parallel, infection of different IN mutants in non-dividing C8166 T cells was also evaluated and similar results were observed (Fig. 4B).

To test whether these IN mutants had similar effects during HIV-1 envelope-mediated single cycle infection, we produced virus stocks by co-transfecting 293T cells with a HIV-1 envelope-competent NLlucΔRI provirus with each Vpr-RT-IN mutant expressor, as described in Materials and Methods. Then, dividing CD4+ C8166 cells were infected with each virus stock (at 10 cpm RT activity/cells). At 48 hours post-infection, cells were collected and measured for luc activity. Results from figure 4C showed that, similar to results obtained from VSV-G pseudotyped virus infection (Fig. 4A), the Luc activity detected in cells infected by HIV-1 envelope competent KK215,9AA, KK240,4AE and RK263,4AA mutant viruses were approximately 13.5%, 6% and 29% of level of D64E mutant infection (Fig. 4C). All of these results confirm the data from HeLa-CD4-CCR5-LTR-β-Gal infection (Fig. 3) by using either VSV-G- and HIV-1 envelope-mediated infections and suggest again that the significantly attenuated infection of KK215,9AA, KK240,4AE and RK263,4AA mutant viruses may be due to their defect(s) at reverse transcription and/or viral DNA nuclear import steps.

### *Effects of IN mutants on reverse transcription, viral DNA nuclear import and integration*

All results so far suggest that these C-terminal mutants might significantly affect early steps during HIV-1 replication. To directly assess the effect of these IN C-terminal mutants on each early step during viral infection, we analyzed the viral DNA synthesis, their nuclear translocation and integration following each IN mutant infection in dividing C8166 cells. Levels of HIV-1 late reverse transcription products were analyzed by semi-quantitative PCR after 12 hours of infection with HIV-1 specific 5'-LTR-U3/3'-Gag primers and Southern blot, as previously described [32,37]. Also, intensity of amplified HIV-1

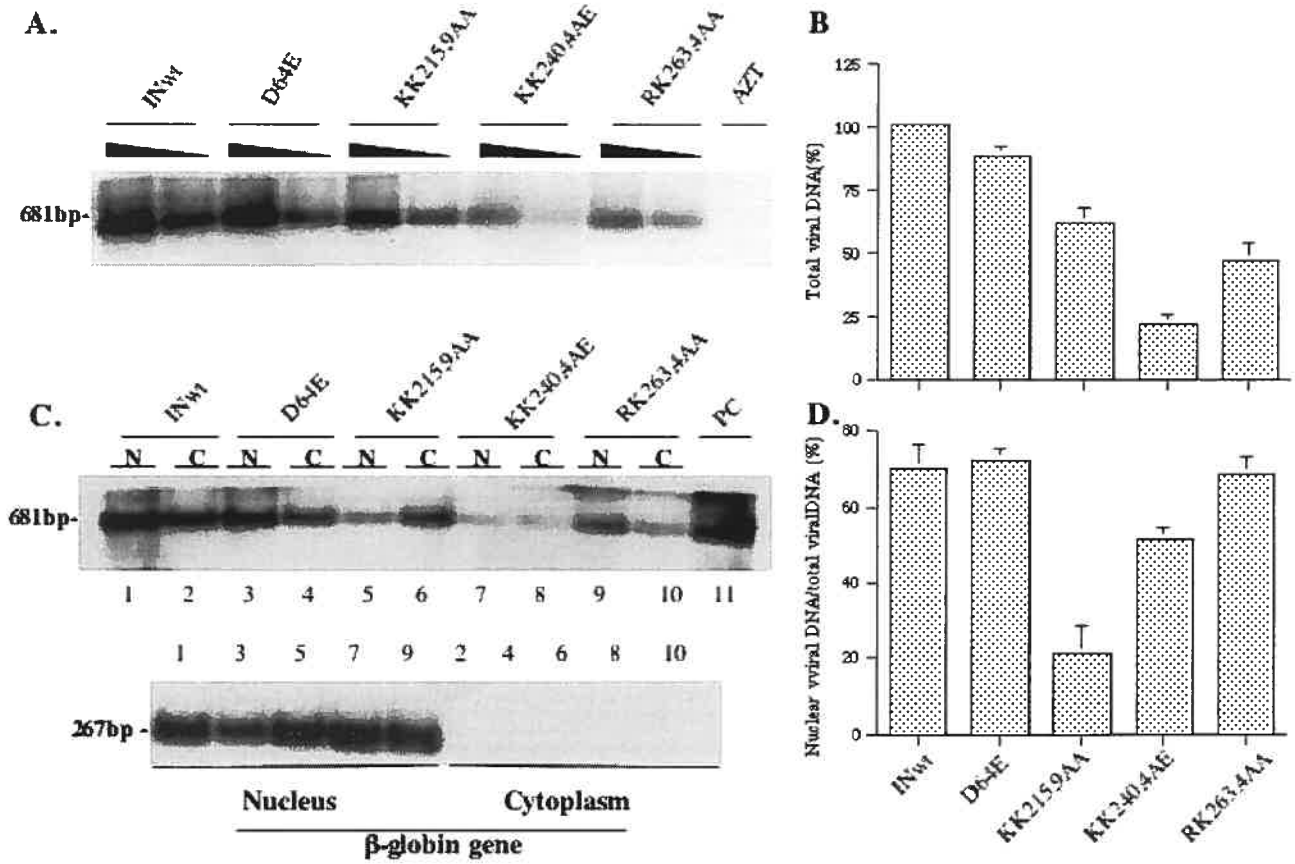


**Figure 5**

**Effects of different IN mutants on HIV-1 reverse transcription and DNA nuclear import.** Dividing C8166 T cells were infected with equal amounts of different HIV-1 IN mutant viruses. A) At 12 hours post-infection, 1 × $10^6$ cells were lysed and the total viral DNA was detected by PCR using HIV-1 LTR-Gag primers and Southern blot. B) Levels of HIV-1 late reverse transcription products detected in panel A were quantified by laser densitometry and viral DNA level of the wt virus was arbitrarily set as 100%. Means and standard deviations from two independent experiments are presented. C) At 24 hours post-infection, 2 × $10^6$ cells were fractionated into cytoplasmic and nuclear fractions as described in Materials and Methods. The amount of viral DNA in cytoplasmic and nuclear fractions were analyzed by PCR using HIV-1 LTR-Gag primers and Southern blot (upper panel, N. nuclear fraction; C. cytoplasmic fraction). Purity and DNA content of each subcellular fraction were monitored by PCR detection of human globin DNA and visualized by specific Southern blot (lower panel). D). The percentage of nucleus-associated viral DNA relative to the total amount of viral DNA for each mutant was also quantified by laser densitometry. Means and standard deviations from two independent experiments are shown.

specific DNA in each sample was evaluated by laser densitometric scanning of bands in Southern blot autoradiograms (Fig. 5A). Results showed that total viral DNA synthesis in both KK215,9AA and RK263,4AA infection reached approximately 61% and 46% of that of the wild type (wt) virus infection (Fig. 5A and 5B). Strikingly, in KK240,4AA sample, detection of viral DNA synthesis was drastically reduced, which only reached 21% of viral DNA level in WT sample (Fig. 5A and 5B). These results indicate that all three C-terminal mutants negatively affected viral reverse transcription during viral infection and KK240,4AA mutant exhibited most profound effect.

Meanwhile, the nucleus- and cytoplasm-associated viral DNA levels were analyzed at 24 hours post-infection in C8166 T cells. The infected cells were first gently lysed and separated into nuclear and cytoplasmic fractions by using a previously described fractionation technique [37]. Then, levels of HIV-1 late reverse transcription products in each fraction were analyzed by semi-quantitative PCR, as

described above. Results revealed differential effects of C-terminal mutants on HIV-1 DNA nuclear import. In the wt, D64E and RK263,4AA virus-infected samples, there were respectively 70%, 72% and 68% of viral DNA associated with nuclear fractions (Fig. 5C (upper panel, lanes 1 and 2; 3 and 4; 9 and 10) and 5D). For KK240,4AE mutant, approximately 51% of viral DNA was nucleus-associated (Fig. 5C (upper panel, lane 7 and 8) and 5D). Remarkably, in KK215,9AA infected sample, viral cDNA was found predominantly in the cytoplasm and only approximately 21% of viral DNA was associated with the nuclear fraction (Fig. 5C (upper panel, lane 5 and 6) and 5D). Meanwhile, the integrity of fractionation procedure was validated by detection of β-globin DNA, which was found solely in the nucleus and levels of this nucleus-associated cellular DNA were similar in each nuclear sample (Fig. 5C, lower panel).

Even though the C-terminal mutants were shown to significantly affect HIV-1 reverse transcription and/or nuclear import, the various low levels of nucleus-associated viral DNA during the early stage of replication (Fig. 5C) may still be accessible for viral DNA integration. To address this question, $1 \times 10^6$ dividing C8166 T cells were infected with equivalent amounts of each single cycle replicating virus stock (5 cpm/cell), as indicated in figure 6 and after 24 hours of infection, the virus integration level was checked by using a previously described sensitive Alu-PCR technique [32], Results revealed that, while the wt virus resulted in an efficient viral DNA integration (Fig. 6, upper panel; lanes 1 and 2), there was no viral DNA integration detected in D64E mutant (lanes 3 to 4) and in all three C-terminal mutant infection samples (lanes 5 to 10), although similar levels of cellular β-globin gene were detected in each sample (Fig. 6, middle panel). These results suggest that, in addition to affecting HIV-1 reverse transcription and nuclear import, all three C-terminal IN mutants tested in this study also negatively affected viral DNA integration. Overall, all of these results indicate that all three IN C-terminal mutants are belonged to class II mutants, which affected different early steps during HIV-1 replication. Among these mutants, the KK240,4AE showed the most profound inhibition on reverse transcription and the KK215,9AA, and to a lesser extent, KK240,4AE, impaired viral DNA nuclear translocation during early HIV-1 infection in C8166 T cells.

## Discussion

In this study, we performed mutagenic studies to analyze different regions in the C-terminal domain of HIV-1 IN that contribute to protein nuclear localization as well as their effects on virus infection. First, our analyses showed that specific lysine mutations introduced in two highly conserved tri-lysine regions in the C-terminal domain of HIV-1 IN impaired protein nuclear accumulation. Second, infection experiments revealed that all three C-terminal mutant viruses (KK215,9AA, KK240,4AE and RK263,4AA) exhibited more severe defect of induction of β-Gal positive cells and luc activity, as compared to an IN class 1 mutant D64E virus, in $CD4^+$ HeLa-β-Gal cells, dividing and non-dividing C8166 T cells. It suggests that all three C-terminal mutant virus infections may have defects at steps prior to integration. Further analysis of total viral DNA synthesis, viral DNA nuclear import and integration indicates that all three C-terminal mutants displayed a class II mutant profile. Even though all of them reduced viral reverse transcription levels, the mutant KK240,4AE showed the most profound inhibitory effect. In addition, the mutant KK215,9AA, and to a lesser extent, KK240,4AE, impaired viral DNA nuclear translocation. These IN mutant-induced defects do not appear to result from various effects of mutants on Gag-Pol processing and maturation given that RT and IN were complemented *in trans* in this HIV-1 single-cycle infection system. Rather, the effect of different IN mutants on reverse transcription and viral DNA nuclear import is likely originated from a role of mutants within the maturing PIC complexes.

Previous work by Gallay *et al.*, have proposed an atypical bipartite NLS ($^{186}$KRK and $^{211}$KELQKQITK) in HIV-1 IN by finding that IN mutants K186Q and Q214/216L lost their karyophilic feature and their ability to bind to karyopherin α *in vitro* [3]. Even though these results were confirmed by Petit and colleagues by studying the intracellular localization of HIV-1 Flag-IN [18], other studies, using GFP-IN fusion protein, did not reveal the importance of K186Q and Q214/216L mutations for HIV-1 IN nuclear localization [16,23,27]. Therefore, the definition of region(s) in HIV-1 IN contributing to the protein nuclear localization is still controversial. In this study, we investigated the intracellular localization of several IN-YFP fusion proteins including the C-terminal-deletion mutant $IN_{1-212}$-YFP, substitution mutants $IN_{KK215,9AA}$-YFP and $IN_{KK240,4AE}$-YFP and found that all of these IN fusion mutants impaired protein nuclear accumulation. It suggests that two C-terminal tri-lysine regions $^{211}$KELQKQITK and $^{236}$KGPAKLLWK contribute to IN nuclear localization. Interestingly, the study by Maertens *et al* also showed that the fusion of HIV-1 IN C-terminal fragment alone with GFP rendered fusion protein to be exclusively in the nucleus, speculating that the C-terminal domain may have a role in HIV-1 nuclear import [28]. However, at this moment, we still could not exclude the possibility that the IN nuclear accumulation could be facilitated by the DNA binding ability of IN protein, as suggested by Devroe *et al* [27]. It has to be noted that two studies have previously observed the nuclear localization of GFP-IN fusion proteins although the C-terminal domain of IN was deleted from the fusion protein [23,28]. It has also been shown that both N-terminal zinc

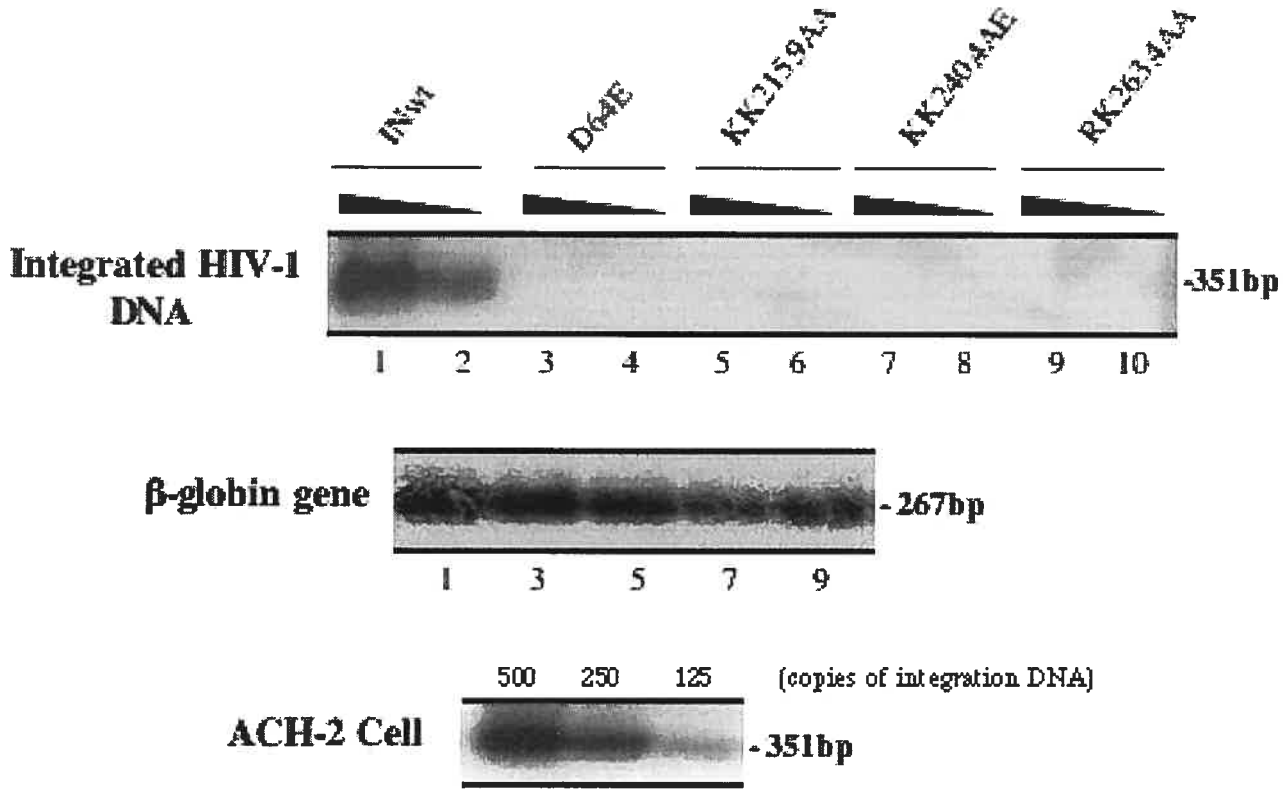


**Figure 6**

**Effect of IN mutants on HIV-1 proviral DNA integration.** Dividing C8166 T cells were infected with equal amounts of different HIV-1 IN mutant viruses. At 24 hours post-infection, 1 × $10^6$ cells were lysed and serial-diluted cell lysates were analyzed by two-step Alu-PCR and Southern blot for specific detection of integrated proviral DNA from infected cells (Upper panel). The DNA content of each lysis sample was also monitored by PCR detection of human β-globin DNA and visualized by specific Southern blot (middle panel). The serial-diluted ACH-2 cell lysates were analyzed for integrated viral DNA and as quantitative control (lower panel). The results are representative for two independent experiments.

binding domain and the central core domain of HIV-1 IN are involved in its interaction with a cellular protein, human lens epithelium-derived growth factor/transcription coactivator p75 (LEDGF/p75) and this IN/LEDGF/p75 interaction is required for GFP-IN nuclear localization [28]. However, our deletion analysis by using IN-YFP fusion protein failed to reveal the importance of both N-terminal and core domains for IN nuclear localization (Fig. 1). One explanation for this discrepancy could be different orientations of fusion proteins used in our study (IN-YFP) and other studies (GFP-IN). It is possible that different forms of fusion proteins may differentially affect the ability of IN to interact with LEDGF/p75 and consequently affect their ability for nuclear targeting. Therefore, it would be interesting to test whether $IN_{KK215,9AA}$-YFP and $IN_{KK240,4AE}$-YFP could loss their ability to interact with LEDGF/p75. These studies are underway.

An important question that needs to be addressed is the impact of nuclear localization-defective IN mutants on HIV-1 replication. Given that most IN mutants characterized so far are classified as class II mutants that cause pleiotropic damage including defects in viral morphogenesis, reverse transcription and integration [16,38], we used a previously described VSV-G pseudotyped HIV-1 RT/IN trans-complement single-cycle replication system [32,39] to minimize differential effects of IN mutants on virus maturation. Also, in our infection experiments, a specific integration-defective class I mutant D64E virus was introduced in order to monitor the viral gene expression from unintegrated HIV-1 DNA species that are already translocated into nucleus during virus infection. It is known that certain levels of selected viral gene expression (*tat and nef*) from unintegrated viral DNA species are detected during this Class I mutant infection [2,35,36]. Interestingly, our

infection analysis revealed that more profound infection defects were found for all three IN C-terminal mutant viruses KK215,9AA, KK240,4AE and RK263,4AA than D64E mutant virus in Hela-CD4-CCR5-β-Gal cells, dividing and non-dividing C8166 T cells (Fig. 3 and 4). These results suggest that these C-terminal IN mutants may affect early steps such as reverse transcription and/or nuclear import and consequently result in a reduced level of viral DNA in the nucleus, which is accessible for *tat* and *nef* expression, To understand the mechanism(s) underlying replication defects of each C-terminal mutant, levels of total reverse transcription were analyzed during early viral infection. Consistent with a previous study [6], infection with D64E mutant virus did not affect reverse transcription as compared to wt virus infection. However, all three C-terminal mutants display various levels of impaired HIV-1 reverse transcription (Fig. 5A and 5B). The mutant KK240,4AE showed strongest inhibition of reverse transcription (21% compared to the wt level (100%)), while mutants KK215,9AA and RK263,4AA reached to 61% and 46% (Fig. 5A and 5B). These data indicate that all of these IN mutants, especially KK240,4AA, negatively affect reverse transcription at early viral infection. Consistently, recent studies have shown that the C-terminal domain of IN contributes to efficient reverse transcription and this domain of IN was able to bind to heterodimeric RT [6,40,41]. It is possible that these C-terminal mutants, especially for KK240,4AE, may disrupt the interaction between IN and RT and result in decreased viral cDNA synthesis.

Subsequently, we examined levels of nucleus- and cytoplasm-associated viral DNA during early virus infection. Results clearly show that the nuclear localization defective mutant KK215,9AA leads to significantly reduced levels of viral DNA in the nucleus, as compared to the wt and D64E viruses (Fig. 5C and 5D). It suggests that the Q region is in fact important for HIV-1 nuclear import. Consistently, a recent study by Lu *et al* also observed that infection of K215A/K219A mutant induced more than 3-fold lower luc activity compared to class I IN mutant D64N/D116N [16]. Moreover, similar to our experimental system, their study revealed that, in the context of VSV-G pseudotyped virus infection in Jurkat cells, 2-LTR circle DNA levels of K215A/K219A and Q214L/Q216L were significantly lower than other mutants V165A and C130G, even though the inhibition of viral reverse transcription mediated by these mutants were comparable [16]. In addition, KK240,4AE mutant also showed a modest impairment of viral DNA nuclear import (Fig. 5C and 5D). In fact, this mutant exhibited the most profound infection defect, compared to other two mutants (KK215,9AA and RK263,4AA) (Fig. 3 and 4). This may be due to combined effects of this mutant on both reverse transcription and viral DNA nuclear import, as shown in Fig. 5. One interesting question is whether such profound infection defect of KK240,4AE mutant virus could be due to a structural alteration by replacing glutamic acid (E) for lysine at position of 244. It seems to be unlikely since 1) the effect of this mutant on nuclear import was not as dramatic as KK215,9AA mutant (as shown in Fig. 5); 2) Wiskerchen *et al* have reported that infection of MAGI cells with two other IN mutants K236A/K240A and K244A/E246A mutants, that are located in the same region as our KK240,4AE mutant, resulted in 0 and 4 β-Gal positive cells, while infection of class I IN mutants produced 700 to 1400 β-Gal positive cells [15]. All of these observations suggest that this region indeed plays an important role for IN activities during early stage of virus infection prior to integration. Also, it has to be noted that although similar inhibition of reverse transcription was seen for KK215,9AA and RK263,4AA mutants, RK263,4AA mutant induced two to three fold higher level of β-Gal positive cells and luc activity than KK215,9AA mutant (Fig. 3 and 4). This is expected since KK215,9AA affected both reverse transcription and nuclear import, while RK263,4AA mutant only impaired reverse transcription (Fig. 5). In addition, our analysis could not detect viral DNA integration in each C-terminal mutant infection (Fig. 6), even though they displayed various low levels of nucleus-associated viral DNA (Fig. 5C). It suggests that these IN mutants may also negatively affect viral integration during their infection. Alternatively, it could be possible that these mutants may have additional defect(s) at an undefined postnuclear entry step that is required for viral DNA integration, as suggested by Lu *et al* [16]. Consistently, their recent reports have shown that several IN mutants in same regions, including K215A/K219A, E244A and R262A/K264A, completely lost virus replication ability in CD4+ Jurkat T cells [16,42].

Up to now, the mechanism(s) underlying the action of HIV-1 IN in viral PIC nuclear import is still unclear. Since IN is a component of viral PIC, at least two factors may affect the contribution of IN to viral PIC nuclear import: first, IN needs to directly or indirectly associate with viral DNA and/or other PIC-associated proteins in order to participate in driving viral DNA into the nucleus; second, IN needs to have a NLS and/or bind to other karyophilic proteins for nuclear translocation. Any mutation disrupting one of these two abilities would affect IN's action for viral DNA nuclear import. A recent study evaluated the effect of several IN core domain mutants targeting key residues for DNA recognition on HIV-1 replication and indicated that, while all of these IN mutants maintained their karyophilic properties, viruses harboring these mutants still severely impaired viral DNA nuclear import [4]. In our study, both KK215,9AA and KK240,4AE mutants clearly lost their karyophilic properties and negatively affected viral DNA nuclear import. However, it is still premature to define

these regions acting as IN NLS, even though a previously described IN mutant Q214/216L, which is also located in proximal tri-lysine domain, has been shown to reduce IN-karyopherin α interaction *in vitro* [3]. More studies are required for further characterization of molecular mechanisms underlying the action of these IN mutants during HIV-1 DNA nuclear import.

## Conclusion

Taken together, the results presented here highlight that all three C-terminal mutants tested in this study resulted in drastic loss of viral infectivity that were due to defects in different early steps of viral replication. Specific lysine mutations introduced in the tri-lysine regions of the C-terminal domain of HIV-1 IN, especially for KK215,9AA, impaired protein nuclear accumulation and HIV-1 PIC nuclear import. Although all of C-terminal mutants inhibited viral reverse transcription to different extents, KK240,4AE mutant exhibited most profound effect on this step. These results suggest that the tri-lysine regions ($^{211}$KELQKQITK and $^{236}$KGPAKLLWK) in the C-terminal of IN are important for HIV-1 reverse transcription and/or nuclear import. More studies are underway to further characterize the mechanisms involved in the action of these regions during early steps of HIV-1 replication.

## Materials and methods

### *Construction of different IN expressors and HIV-1 RT/IN defective provirus*

The full-length wild-type HIV-1 IN cDNA was amplified by polymerase chain reaction (PCR) using HIV-1 HxBru strain [30] as template and an engineered initiation codon (ATG) was placed prior to the first amino acid (aa) of IN. The primers are 5'-IN-HindIII-ATG (5'-GCGCAAGCTTGGATAGATGTTTTTAGATGGAA-3') and 3'-IN-Asp718 (5'-CCATGTGTGGTACCTCATCCTGCT-3'). The PCR product was digested with *Hind*III and *Asp*718 restriction enzymes and cloned in frame to 5' end of EYFP cDNA in a pEYFP-N1 vector (BD Biosciences Clontech) and generated a IN-YFP fusion expressor. Also, cDNA encoding for truncated IN (aa 50 to 288 or aa 1 to 212) was amplified by PCR and also cloned into pEYFP-N1 vector. The primers for generation of IN50-288 cDNA are IN50-HindIII-ATG-5'(5'– GCGCAAGCTTGGATAGATGCATGGACAAGTAG-3) and 3'-IN-Asp718 and primers for amplifying IN1-212 cDNA are IN-HindIII-ATG-5' and IN-212-XmaI-3'(5'-CAATTCCCGGGTTTGTATGTCTGTTTGC-3). IN substitution mutants $IN_{KK215,9AA}$-YFP, $IN_{KK240,4AE}$-YFP and $IN_{RK263,4AA}$-YFP, were generated by a two-step PCR-based method [43] by using a 5'-primer (5'-IN-HindIII-ATG), a 3'-primer (3'-IN-Asp718) and complementary primers containing desired mutations. Amplified IN cDNAs harboring specific mutations were then cloned into pEYFP-N1 vector. To improve the expression of each IN-YFP fusion protein, all IN-YFP fusing cDNAs were finally subcloned into a SVCMV vector, which contains a cytomegalovirus (CMV) immediate early gene promoter [43].

To construct HIV-1 RT/IN defective provirus NLlucΔBglΔRI, we used a previously described HIV-1 envelope-deleted NLlucΔBglD64E provirus as the backbone (kindly provided by Dr. Irvin S.Y. Chen). In this provirus, the *nef* gene was replaced by a firefly luciferase gene [33]. The *Apa*I/*Sal*I cDNA fragment in NLlucBglD64E was replaced by the corresponding fragment derived from a HIV-1 RT/IN deleted provirus R$^-$/ΔRI [32] and generated a RT/IN deleted provirus NLlucΔBglΔRI, in which RT and IN gene sequences were deleted while a 194-bp sequence harboring cPPT/CTS *cis*-acting elements was maintained. To restore HIV-1 envelope gene sequence in NLlucΔBglΔRI provirus, the *Sal*I/*BamH*I cDNA fragment in this provirus was replaced by a corresponding cDNA fragment from a HIV-1 envelope competent provirus R$^-$/ΔRI [32] and the resulting provirus is named as NLlucΔRI. To functionally complement RT/IN defects of NLlucΔBglΔRI, a CMV-Vpr-RT-IN fusion protein expressor [32] was used in this study. Co-transfection of NLlucΔBglΔRI, CMV-Vpr-RT-IN and a vesicular stomatitis virus G (VSV-G) glycoprotein expressor results in the production of VSV-G pseudotyped HIV-1 that can undergo for single cycle replication in different cell types [32]. To investigate the effect of IN mutants on viral replication, different mutants KK215,9AA, KK240.4AE, RK263,4AA or D64E were introduced into CMV-Vpr-RT-IN expressor by PCR-based method as described above and using a 5'-primer corresponding to a sequence in RT gene and including a natural *Nhe*I site (5'-GCAGCTAGCAGGGAGACTAA-3'), a 3'-primer (3'-IN-stop-*Pst*I, 5'– CTGTTCCTGCAGCTAATCCTCATCCTG-3') and the complementary oligonucleotide primers containing desired mutations. All IN mutants were subsequently analyzed by DNA sequencing to confirm the presence of mutations or deletions.

### *Cell lines and reagents*

Human embryonic kidney 293T, HeLa and HeLa-CD4-CCR5-β-Gal cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Human C8166 T-lymphoid cells were maintained in RPMI-1640 medium. Antibodies used in the immunofluorescent assay, immunoprecipitation or western blot are as follows: The HIV-1 positive human serum 162 and anti-HIVp24 monoclonal antibody used in this study were previously described [44]. The rabbit anti-GFP and anti-IN antibodies were respectively obtained from Molecular Probes Inc and through AIDS Research Reference Reagent Program, Division of AIDS, NIAID, NIH. Aphidicolin was obtained from Sigma Inc.

***Cell transfection and immunofluorescence assay***

DNA transfection in 293T and HeLa cells were performed with standard calcium phosphate DNA precipitation method. For immunofluorescence analysis, HeLa cells were grown on glass coverslip (12 $mm^2$) in 24-well plate. After 48 h of transfection, cells on the coverslip were fixed with PBS-4% paraformaldehyde for 5 minutes, permeabilized in PBS-0.2% Triton X-100 for 5 minutes and incubated with primary antibodies specific for GFP or HIV-1 IN followed by corresponding secondary FITC-conjugated antibodies. Then, cells on the coverslip were viewed using a computerized Axiovert 200 inverted fluorescence microscopy (Becton Deckson Inc).

***Virus production and infection***

Production of different single-cycle replicating virus stocks and measurement of virus titer were previously described [32]. Briefly, 293T cells were co-transfected with RT/IN defective NLlucΔBglΔRI provius, a VSV-G expressor and each of CMV-Vpr-RT-IN (wt/mutant) expressor. To produce HIV-1 envelope competent single cycle replicating virus, 293T cells were co-transfected with NLlucΔRI and different CMV-Vpr-RT-IN (wt/mutant) expressors. After 48 hours of transfection, supernatants were collected and virus titers were quantified by RT activity assay [43].

To test the effect of IN mutants on virus infection, equal amounts of virus were used to infect HeLa-CCR5-CD4-β-Gal cells, dividing and non-dividing C8166 T cells. To compare the infection of each viral stock in HeLa-CCR5-CD4-β-Gal cells, numbers of infected cells (β-Gal positive cells) were evaluated by the MAGI assay 48 hours post-infection (p.i) as described previously [34]. To infect CD4+ T cells, dividing or aphidicolin-treated non-dividing C8166 T cells (with 1.3 μg/ml of aphidicolin) were infected with equivalent amounts of single cycle replicating viruses (5 cpm/cell) for 2 hours. Then, infected cells were washed and cultured in the absence or presence of the same concentration of aphidicolin. At 48 hours post-infection, 1 × $10^6$ cells from each sample were collected, washed twice with PBS, lysed with 50 μl of luciferase lysis buffer (Fisher Scientific Inc) and then, 10 μl of cell lysate was subjected to the luciferase assay by using a Top-Count®NXT™ Microplate Scintillation & Luminescence Counter (Packard, Meriden) and the luciferase activity was valued as relative luciferase units (RLU). Each sample was analyzed in duplicate and the average deviation was calculated.

***Immunoprecipitation and Western blot analyses***

For detection of IN-YFP fusion proteins, 293T cells transfected with each IN-YFP expressor were lysed with RIPA lysis buffer and immunoprecipitated using human anti-HIV serum. Then, immunoprecipitates were run in 12% SDS-PAGE and analyzed by Western blot using rabbit anti-GFP antibody. To analyze virion-incorporation of IN and virus composition, 293T cells were co-transfected with NLlucΔBglΔRI provirus and each of CMV-Vpr-RT-IN (wt/mutant) expressors. After 48 hours, viruses were collected, lysed with RIPA lysis buffer and immunoprecipitated with human anti-HIV serum. Then, immunoprecipitates were run in 12% SDS-PAGE and analyzed by Western blot with rabbit anti-IN antibody and anti-p24 monoclonal antibody or anti-HIV serum.

***HIV-1 reverse-transcribed and integrated DNA detection by PCR and Southern blotting***

C8166 T cells were infected with equal amount of the wt or IN mutant viruses for 2 hours, washed for three times and cultured in RPMI medium. To detect total viral DNA synthesis, at 12 hours post-infection, equal number (1 × $10^6$ cells) of cells were collected, washed twice with PCR washing buffer (20 mM Tris-HCl, pH8.0, 100 mM KCl), and lysed in lysis buffer (PCR washing buffer containing 0.05% NP-40, 0.05% Tween-20). Lysates were then incubated at 56°C for 30 min with proteinase K (100 μg/ml) and at 90°C for 10 min prior to phenol-chloroform DNA purification. To detect viral cDNA from each sample, all lysates were serially diluted 5-fold and subjected to PCR analysis. The primers used to detect late reverse transcription products were as following: 5'-LTR-U3, 5'-GGATGGTGCTTCAAGCTAGTACC-3' (nt position 8807, +1 = start of BRU of transcription initiation); 3'-Gag 5'-ACTGACGCTCTCGCACCCATCTCTCTC-3' (nt position 329). The probe for southern blot detection was generated by PCR with a 5'-LTR-U5 oligonucleotide, 5'-CTCTAGCAGTGGCGCCCGAACAGGGAC-3' (nt position 173) and the 3'-Gag oligo. PCR was carried out using 1× HotStar Taq Master Mix kit (QIAGEN, Mississauga, Ontario), as described previously [32].

To analyze nucleus- and cytoplasm-associated viral DNA, a subcellular fractionation of infected C8166 T cells (2 × $10^6$) was performed after 24 hours of infection, as described previously [37]. Briefly, infected cells were pelleted and resuspended in ice-cold PCR lysis buffer (washing buffer containing 0,1% NP-40). After a 5-min incubation on ice, the nucleus was pelleted by centrifugation, washed twice with PCR wash buffer, and lysed in lysis buffer (0,05% NP-40, 0,05% Tween-20). Then, both cytoplasmic sample (supernatant from the first centrifugation) and the nuclear sample were treated with proteinase K and used for PCR analysis, as described above.

Integrated proviral DNA was detected in cell lysates by a modified nested Alu-PCR [32], in which following the first PCR, a second PCR was carried-out to amplify a portion of the HIV-1 LTR sequence from the first Alu-LTR PCR-amplified products. The first PCR was carried out by

using primers including 5'-Alu oligo (5'-TCCCAGCTACTCGGGAGGCTGAGG-3') and 3'-LTR oligo (5'-AGGCAAGCTTTATTGAGGGCTTAAGC-3') (nt position 9194) located respectively in the conserved region of human Alu sequence and in HIV-1 LTR. The primer used for both of the second nested PCR and for generating a probe are 5'-NI: 5'-CACACACAAGGCTACT-TCCCT-3' and 3'-NI: 5'-GCCACTCCCCAGTCCCGCCC-3'. As a control, the first and second PCR primer pairs were also used in parallel to detect integrated viral DNA from serially diluted ACH-2 cells, which contain one viral copy/cell, in a background of uninfected C8166 cellular DNA.

To evaluate the DNA content of extracted chromosomal DNA preparations, detection of human β-globin gene was carried-out by PCR, as described previously [37]. All final PCR products were electrophoresed through 1.2% agarose gel and transferred to hybridization transfer membrane (GeneScreen Plus, PerkinElmer Life Sciences), subjected to Southern hybridization by using specific PCR DIG-Labeling probes (Roche Diagnostics, Laval, Que) and visualized by a chemiluminescent method. Densitometric analysis was performed using a Personal Molecular Imager (Bio-Rad) and Quantity One software version 4.1.

## Authors' contributions

Z-J Ao designed and performed experiments, constructed most IN mutants and wrote the manuscript. KR Fowke provided technique support and critically evaluated the manuscript. EA Cohen participated in the design of the study and critically evaluated the manuscript. X-J Yao designed the study and coordinated it. All authors read and approved the final manuscript.

## Acknowledgements

We would like to thank Nicole Rougeau, John Rutherford and Andres Finzi for their technical support. We also thank Dr. Irvin S.Y. Chen for kindly providing NLlucBglD64E provirus and Dr. Kevin Coombs for critical reading of the manuscript. We are also grateful to Drs. M. Emerman and D. Grandgenett for the HeLa-CD4-CCR5-β-Gal cells and anti-IN antiserum that were obtained through the AIDS Research Reference Reagent Program, Division of AIDS, NIAID, NIH. Eric A. Cohen is the recipient of the Canada Research Chair in Human Retrovirology. This work was supported by a Canadian Institutes of Health Research (CIHR) grant (HOP-63013) to X.J.Y.

## References

1. Bukovsky A, Gottlinger H: **Lack of integrase can markedly affect human immunodeficiency virus type 1 particle production in the presence of an active viral protease.** *J Virol* 1996, **70**:6820-6825.
2. Engelman A, Englund G, Orenstein JM, Martin MA, Craigie R: **Multiple effects of mutations in human immunodeficiency virus type 1 integrase on viral replication.** *J Virol* 1995, **69**:2729-2736.
3. Gallay P, Hope T, Chin D, Trono D: **HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway.** *Proc Natl Acad Sci U S A* 1997, **94**:9825-9830.
4. Ikeda T, Nishitsuji H, Zhou X, Nara N, Ohashi T, Kannagi M, Masuda T: **Evaluation of the functional involvement of human immunodeficiency virus type 1 integrase in nuclear import of viral cDNA during acute infection.** *J Virol* 2004, **78**:11563-11573.
5. Nakamura T, Masuda T, Goto T, Sano K, Nakai M, Harada S: **Lack of infectivity of HIV-1 integrase zinc finger-like domain mutant with morphologically normal maturation.** *Biochem Biophys Res Commun* 1997, **239**:715-722.
6. Wu X, Liu H, Xiao H, Conway JA, Hehl E, Kalpana GV, Prasad V, Kappes JC: **Human immunodeficiency virus type 1 integrase protein promotes reverse transcription through specific interactions with the nucleoprotein reverse transcription complex.** *J Virol* 1999, **73**:2126-2135.
7. Piller SC, Caly L, Jans DA: **Nuclear import of the pre-integration complex (PIC): the Achilles heel of HIV?** *Curr Drug Targets* 2003, **4**:409-429.
8. Lee SP, Xiao J, Knutson JR, Lewis MS, Han MK: **Zn2+ promotes the self-association of human immunodeficiency virus type-1 integrase in vitro.** *Biochemistry* 1997, **36**:173-180.
9. Ellison V, Gerton J, Vincent KA, Brown PO: **An essential interaction between distinct domains of HIV-1 integrase mediates assembly of the active multimer.** *J Biol Chem* 1995, **270**:3320-3326.
10. Heuer TS, Brown PO: **Photo-cross-linking studies suggest a model for the architecture of an active human immunodeficiency virus type 1 integrase-DNA complex.** *Biochemistry* 1998, **37**:6667-6678.
11. Engelman A, Craigie R: **Identification of conserved amino acid residues critical for human immunodeficiency virus type 1 integrase function in vitro.** *J Virol* 1992, **66**:6361-6369.
12. van Gent DC, Groeneger AA, Plasterk RH: **Mutational analysis of the integrase protein of human immunodeficiency virus type 2.** *Proc Natl Acad Sci U S A* 1992, **89**:9598-9602.
13. Eijkelenboom AP, Lutzke RA, Boelens R, Plasterk RH, Kaptein R, Hard K: **The DNA-binding domain of HIV-1 integrase has an SH3-like fold.** *Nat Struct Biol* 1995, **2**:807-810.
14. Lutzke RA, Plasterk RH: **Structure-based mutational analysis of the C-terminal DNA-binding domain of human immunodeficiency virus type 1 integrase: critical residues for protein oligomerization and DNA binding.** *J Virol* 1998, **72**:4841-4848.
15. Wiskerchen M, Muesing MA: **Human immunodeficiency virus type 1 integrase: effects of mutations on viral ability to integrate, direct viral gene expression from unintegrated viral DNA templates, and sustain viral propagation in primary cells.** *J Virol* 1995, **69**:376-386.
16. Lu R, Limon A, Devroe E, Silver PA, Cherepanov P, Engelman A: **Class II integrase mutants with changes in putative nuclear localization signals are primarily blocked at a postnuclear entry step of human immunodeficiency virus type 1 replication.** *J Virol* 2004, **78**:12735-12746.
17. Cannon PM, Byles ED, Kingsman SM, Kingsman AJ: **Conserved sequences in the carboxyl terminus of integrase that are essential for human immunodeficiency virus type 1 replication.** *J Virol* 1996, **70**:651-657.
18. Petit C, Schwartz O, Mammano F: **The karyophilic properties of human immunodeficiency virus type 1 integrase are not required for nuclear import of proviral DNA.** *J Virol* 2000, **74**:7119-7126.
19. Bouyac-Bertoia M, Dvorin JD, Fouchier RA, Jenkins Y, Meyer BE, Wu LI, Emerman M, Malim MH: **HIV-1 infection requires a functional integrase NLS.** *Molecular Cell* 2001, **7**:1025-1035.
20. Depienne C, Mousnier A, Leh H, Rouzic EL, Dormont D, Benichou S, Dargemont C: **Characterization of the nuclear import pathway for HIV-1 integrase.** *J Biol Chem* 2001, **276**:18192-18107.
21. Petit C, Schwartz O, Mammano F: **Oligomerization within virions and subcellular localization of human immunodeficiency virus type 1 integrase.** *J Virol* 1999, **73**:5079-5088.
22. Pluymers W, Cherepanov P, Schols D, Clercq ED, Debyser Z: **Nuclear localization of human immunodeficiency virus type 1 integrase expressed as a fusion protein with green fluorescent protein.** *Virology* 1999, **258**:327-332.
23. Tsurutani N, Kubo M, Maeda Y, Ohashi T, Yamamoto N, Kannagi M, Masuda T: **Identification of critical amino acid residues in human immunodeficiency virus type 1 IN required for efficient proviral DNA formation at steps prior to integration in dividing and nondividing cells.** *J Virol* 2000, **74**:4795-4806.
24. Dvorin JD, Bell P, Maul GG, Yamashita M, Emerman M, Malim MH: **Reassessment of the roles of integrase and the central DNA**

**flap in human immunodeficiency virus type 1 nuclear import.** *J Virol* 2002, **76:**12087-12096.
25. Limon A, Devroe E, Lu R, Ghory HZ, Silver PA, Engelman A: **Nuclear localization of human immunodeficiency virus type 1 preintegration complexes (PICs): V165A and R166A are pleiotropic integrase mutants primarily defective for integration, not PIC nuclear import.** *J Virol* 2002, **76:**10598-10607.
26. Kukolj G, Jones MS, Skalka M: **Subcellular localization of avian sarcoma virus and human immunodefiiency virus type 1 integrase.** *J Virol* 1997, **71:**843-847.
27. Devroe E, Engelman A, Silver PA: **Intracellular transport of human immunodeficiency virus type 1 integrase.** *J Cell Sci* 2003, **116:**4401-4408.
28. Maertens G, Cherepanov P, Pluymers W, Busschots K, De Clercq E, Debyser Z, Engelborghs Y: **LEDGF/p75 is essential for nuclear and chromosomal targeting of HIV-1 integrase in human cells.** *J Biol Chem* 2003, **278:**33528-33539.
29. Llano M, Vanegas M, Fregoso O, Saenz D, Chung S, Peretz M, Poeschla EM: **LEDGF/p75 determines cellular trafficking of diverse lentiviral but not murine oncoretroviral integrase proteins and is a component of functional lentiviral preintegration complexes.** *J Virol* 2004, **78:**9524-9537.
30. Yao XJ, Subbramanian RA, Rougeau N, Boisvert F, Bergeron D, Cohen EA: **Mutagenic analysis of human immunodeficiency virus type 1 Vpr: role of a predicted N-terminal alpha-helical structure in Vpr nuclear localization and virion incorporation.** *J Virol* 1995, **69:**7032-7044.
31. Kuiken C, Foly B, Hahn B, Marx P, McCutchan F, Mellors J, Wolinsky SM, Korber B: **HIV Sequence Compendium 2001.** *Los Alamos National Laboratory* 2001.
32. Ao Z, Yao X, Cohen EA: **Assessment of the role of the central DNA flap in human immunodeficiency virus type 1 replication by using a single-cycle replication system.** *J Virol* 2004, **78:**3170-3177.
33. Poon B, Chen IS: **Human immunodeficiency virus type 1 (HIV-1) Vpr enhances expression from unintegrated HIV-1 DNA.** *J Virol* 2003, **77:**3962-3972.
34. Kimpton J, Emerman M: **Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated b-galactosidase gene.** *J Virol* 1992, **66:**2232-2239.
35. Brussel A, Sonigo P: **Evidence for gene expression by unintegrated human immunodeficiency virus type 1 DNA species.** *J Virol* 2004, **78:**11263-11271.
36. Wu Y, Marsh JW: **Selective transcription and modulation of resting T cell activity by preintegrated HIV DNA.** *Science* 2001, **293:**1503-1506.
37. Simon JHM, Malim MH: **The human immunodeficiency virus type 1 Vif protein modulates the postpenetration stability of viral nucleoprotein complexes.** *J Virol* 1996, **70:**5297-5305.
38. Nakajima N, Lu R, Engelman A: **Human immunodeficiency virus type 1 replication in the absence of integrase-mediated dna recombination: definition of permissive and nonpermissive T-cell lines.** *J Virol* 2001, **75:**7944-7955.
39. Wu X, Liu H, Xiao H, Conway JA, Hunter E, Kappes JC: **Functional RT and IN incorporated into HIV-1 particles independently of the Gag/Pol precursor protein.** *Embo J* 1997, **16:**5113-5122.
40. Hehl EA, Joshi P, Kalpana GV, Prasad VR: **Interaction between human immunodeficiency virus type 1 reverse transcriptase and integrase proteins.** *J Virol* 2004, **78:**5056-5067.
41. Zhu K, Dobard C, Chow SA: **Requirement for integrase during reverse transcription of human immunodeficiency virus type 1 and the effect of cysteine mutations of integrase on its interactions with reverse transcriptase.** *J Virol* 2004, **78:**5045-5055.
42. Lu R, Ghory HZ, Engelman A: **Genetic analyses of conserved residues in the carboxyl-terminal domain of human immunodeficiency virus type 1 integrase.** *J Virol* 2005, **79:**10356-10368.
43. Yao XJ, Kobinger G, Dandache S, Rougeau N, Cohen E: **HIV-1 Vpr-chloramphenicol acetyltransferase fusion proteins: sequence requirement for virion incorporation and analysis of antiviral effect.** *Gene Ther* 1999, **6:**1590-1599.
44. Yao XJ, Mouland AJ, Subbramanian RA, Forget J, Rougeau N, Bergeron D, Cohen EA: **Vpr stimulates viral expression and induces cell killing in human immunodeficiency virus type 1-infected dividing Jurkat T cells.** *J Virol* 1998, **72:**4686-4693.