Functional characterization of HIV-1 integrase in yeast and during viral replication

By

Zaikun Xu

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

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Canada

THE UNIVERSITY OF MANITOBA

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ACKNOWLEDGEMENTS

Firstly, I would like to express my deep and sincere gratitude to my supervisor, Dr. Xiaojian Yao, for offering me the opportunity to work in his laboratory. Since I started my MSc assignment in 2005, I have known Yao as a sympathetic and principle-centered person. His overly enthusiasm on research and his mission for providing high-quality work, has made a deep impression on me. I owe him lots of gratitude for showing me this way of research. His wide knowledge, logical way of thinking, understanding, encouraging and personal guidance has provided a good basis for the present thesis. Besides of being an excellent supervisor, Yao is as close as a good friend to me. I am really glad that I have come to get know him in my life.

Secondly, I wish to express my warm and sincere thanks to my supervisory committee members including Dr. Kevin Coombs and Dr. Sam Kung for their support and guidance during my Master career. Their ideals and expertise have made a remarkable contribution to my project.

In addition, I also wish to extend my gratitude to laboratory members including Zhujun Ao, Guanyou Huang, Meaghan Labine, Yuanyuan Gu, and Lina Wang for their time, assistance, and guidance.

Finally, I would like to give my special thanks to my wife, Ran Chen. Words fail me to express my appreciation to her whose patient love, persistent confidence in me, and selfless efforts to the family has taken the load off my shoulder. I want to thank my wife for all the support that she gave me during the years I have been working on this thesis. Without her constant help and sacrifice this thesis would not have been completed.

Overall, I would like to thank everybody who was important to the successful

realization of this thesis, as well as expressing my apology that I could not mention personally one by one.

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ABBREVIATIONS

APOBEC3G	Apolipoprotein B mRNA-editing Enzyme-Catalytic Polypeptide-like 3G
AIDS	Acquired Immune Deficiency Syndrome
BAF	Barrier-to-Autointegration Factor
CA	Capsid
CCD	Catalytic Core Domain
CTD	C-terminal Domain
CypA	Cyclophilin A
DMEM	Dulbecco's Modified Eagle's Medium
ECL	Enhanced Chemiluminescence
Env	Envelope glycoprotein
FCS	Fetal Calf Serum
Gag	Group-specific antigen
HAART	Highly Active Anti-Retroviral Therapy
HIV-1	Human Immunodeficiency Virus Type 1
HMGI (Y)	High Mobility Group Protein Y
IBD	Integrase Binding domain
IN	Integrase
INI1	Integrase Interactor 1
IP	Immunoprecipitation
LEDGF/p75	The human lens epithelium-derived growth factor p75
LTR	Long Terminal Repeat
MA	Matrix
MHC I	Major Histocompatibility Class I
NC	Nucleocapsid
Nef	Negative factor
NF-κB	Nuclear Transcription factor κB
NLS	Nuclear Localization Signal

NPC	Nuclear Pore Complex
NTD	N-terminal domain
PBMC	Peripheral Blood Mononuclear Cells
PIC	Preintegration Complex
Pol	Polymerase protein
PR	Protease
RLU	Relative Light Unit
RQ-PCR	Real-time Quantitative Polymerase Chain Reaction
RRE	Rev Responsive Element
RT	Reverse transcriptase
RTC	Reverse-transcription complex
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
VSV-G	Vesicular Stomatitis Virus Envelop Glycoprotein
WB	Western Blot assay
YFP	Yellow- fluorescent protein

ABSTRACT

INTRODUCTION: Human immunodeficiency virus type 1 (HIV-1) integrase (IN) is the key viral enzyme to catalyze the integration of proviral cDNA into the host genome, an essential step during viral replication. In addition to catalyzing the integration reaction, HIV-1 IN also plays important roles for other early steps of viral replication, including viral reverse transcription and viral DNA nuclear import. Even though extensive research has been tried to further understand how IN contributes to HIV-1 integration, the detailed mechanism underlying its action is still not fully understood. One major reason influencing the integration study could be due to the multiple effects of IN on other replication steps prior to integration such as reverse transcription and nuclear import. To facilitate the integration study *in vivo* without being influenced by other functions of IN, the HIV-1 IN yeast expressing system was established and found that expression of functional IN in some *Saccharomyces cerevisiae* yeast strains led to the emergence of a lethal phenotype. This IN-induced yeast lethality could be closely correlated with the integration reaction and the yeast system may be a useful tool to study the HIV-1 integration process and to screen drugs capable of inhibiting HIV-1 integration *in vivo*. In this study, we have performed mutagenic analyses to further delineate the critical amino acid(s) and/or motif(s) in HIV-1 IN responsible for the lethality in a HP16 yeast strain. Also, we did functional and biochemical analyses to investigate how these different IN mutants identified from the yeast lethal phenotype system affect viral replication and the mechanism involved in replication defectiveness.

METHODS, RESULTS AND CONCLUSIONS: A panel of IN mutants which specifically target different regions of IN were introduced into a yeast expressing

system to test their lethal activity in yeast strain HP16. Results clearly revealed that three mutants that harbour C-terminal mutations in the catalytic core domain of HIV-1 IN (V165A, A179P and KR186,7AA) lost their lethal activity. However, the IN catalytic mutants D64E, D116A and D64E/D116A still remained active for this IN activity in yeast. To investigate the effects of three lethal phenotype defective IN mutants (V165A, A179P and KR186,7AA) on viral replication, we introduced these IN mutants into vesicular stomatitis virus envelop glycoprotein (VSV-G) pseudotyped HIV-1 strains. Our results clearly showed that all three VSV-G pseudotyped viruses were unable to mediate infection in CD4+ C8166 cells. Interestingly, their lack of infectivity was shown to be partially complemented by the D64E IN mutant, indicating that the infectivity defect of these viruses was distinguishable from the catalytic defects of D64E during viral replication. Moreover, we further demonstrated that, unlike the wild type IN and D64E mutant, the three yeast lethal phenotype-defective mutants (V165A, A179P and KR186,7AA) lost their ability to bind to cellular chromatin. Taken together, these results provide evidence that the chromatin binding ability mediated by the C-terminal region of the catalytic core domain of HIV-1 IN plays an important role in the lethal activity of the enzyme in yeast as well as in proviral DNA integration during HIV-1 infection. Further investigation of the critical motif(s) of IN for chromatin binding ability may serve as the basis for the discovery of new class of anti-IN agents.

CHAPTER 1 INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) belongs to the *Lentiviridae* genus of retroviruses and is the cause of acquired immunodeficiency syndrome, AIDS. Since it was first recognized in 1981, AIDS has killed more than 25 million people in the world, making it one of the most destructive diseases in recent history. Currently, HIV-1 infection in humans is pandemic. In 2005 alone, an estimated 2.4–3.3 million people lost their lives to AIDS, of which more than 570,000 were children. It is estimated that about 0.6% of the world's living population is infected with HIV (2006 report on the global AIDS epidemic).

HIV-1 primarily infects the human immune system such as helper T cells (specifically $CD4^+$ T cells), macrophages and dendritic cells. HIV infection decreases the levels of $CD4^+$ T cells and once $CD4^+$ T cell numbers decline below a critical level, cell-mediated immunity will be lost, and then the human body becomes progressively more susceptible to opportunistic infections. If untreated, eventually most HIV-infected individuals will develop AIDS and then die of the subsequent infections.

Currently, there is no vaccine or cure for HIV or AIDS. The only known method of prevention is avoiding exposure to the virus. However, current treatment strategies for HIV-1 infection, known as highly active antiretroviral therapy (HAART) which targets the viral enzymes reverse transcriptase (RT) and protease (PR) has substantially reduced the death rate from AIDS in those areas where these drugs are widely available (Palella et al., 1998). However, these anti-retroviral drugs are so expensive that the majority of the world's infected individuals do not have access to medications and treatments for AIDS. Moreover, even though these drugs can delay the progression of the disease, current

therapy is not able to eradicate the virus and readily leads to the emergence of drug-resistant HIV strains (Hertogs et al., 2000; Miller and Larder, 2001). Thus, the identification of additional targets and the development of new classes of antiviral compounds are essential to increase the potency of inhibition and to prevent the development of resistance.

In addition to RT and PR, another HIV-1 enzymatic molecule, integrase (IN), is also an attractive target for the development of novel inhibitors (Tarrago-Litvak et al., 2002). Since HIV-1 IN plays multiple roles during viral replication, each IN-involved viral replication step can be targeted respectively for blocking virus infection. Indeed, extensive studies have been made to elucidate the mechanisms involved in IN's action at different steps of viral replication including reverse transcription, nuclear import, and integration. Moreover, specific interactions between HIV-1 IN and its cellular partners also could be the target to develop novel inhibitors which may have the potential to become new therapeutic agents.

In this chapter, the HIV-1 virion structure, genomic organization as well as HIV-1 replication cycle will be briefly described. Afterwards, the literature review and discussion will be primarily focused on HIV-1 IN's structures and functions especially for its role during the integration reaction. Finally, this chapter will end with functional investigation of HIV-1 IN in yeast.

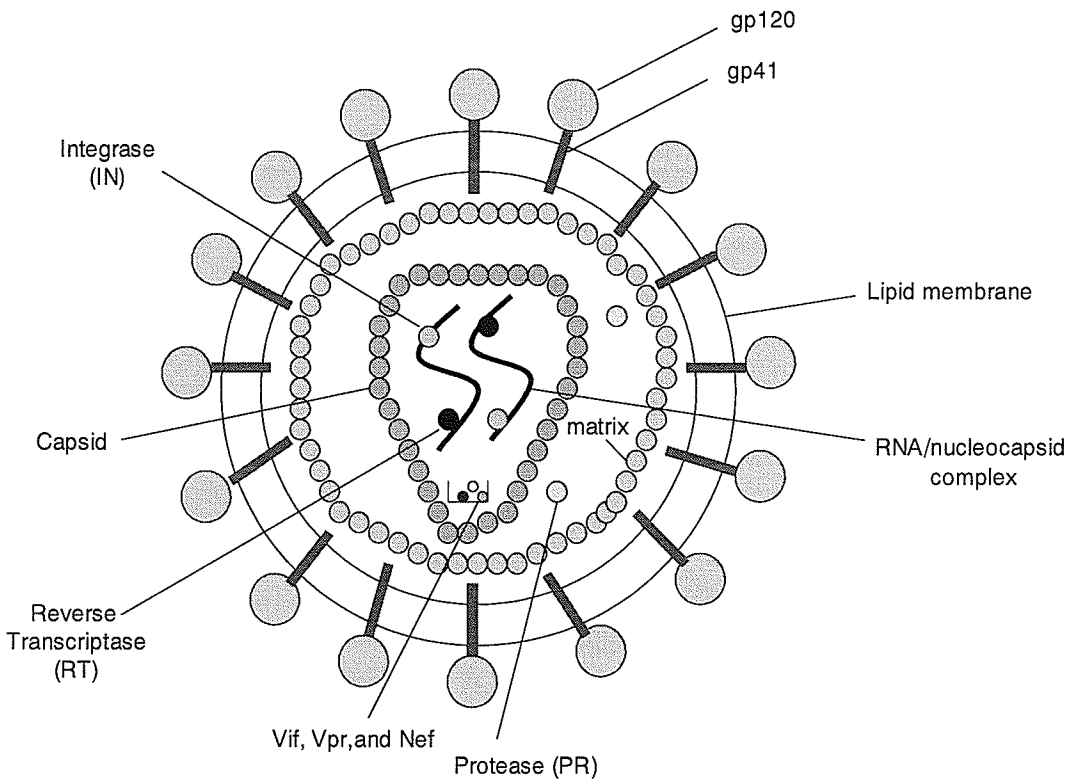
1.1 HIV-1 virion and genome

HIV-1 is classified as a lentivirus in a subgroup of retroviruses. The mature virion is an enveloped, roughly spherical particle and is about 120 nm in diameter. It is composed of two copies of positive single-stranded RNA genome enclosed by a conical

capsid comprising the viral protein, p24. The single-stranded RNA is tightly bound to nucleocapsid proteins, p7 and enzymes which are indispensable for the formation of infectious viruses such as reverse transcriptase (RT) and integrase (IN). Some other regulatory and accessory viral proteins including Vif, Vpr, and Nef are also enclosed in the capsid (Fig.1.1A). A matrix composed of the viral protein p17 surrounds the capsid ensuring the integrity of the virion particle. This is in turn surrounded by the viral envelope. The envelope is derived from the plasma membrane of an infected human cell when a newly formed virus particle buds from the cell. The envelope includes the glycoproteins gp120 and gp41 (Fig.1.1A).

The HIV-1 genome is encoded by a 9 kb single-stranded RNA molecule and contains 9 open reading frames (Fig.1.1B). The largest three reading frames respectively transcribe the Gag, Pol, and Env polyproteins, which encode the structural proteins for new virus particles and are proteolytically processed into proteins common to all members of the retrovirus family. Gag is processed into matrix (MA), capsid (CA), nucleocapsid (NC), and p6 which makes up the inner core of the viral particle. Env polyprotein derived gp120 (surface, SU) and gp41 (transmembrane, TM) make up the virus's outer membrane proteins. This glycoprotein complex enables the virus to attach to and fuse with target cells to initiate the replication cycle. The polyprotein Pol includes the viral enzymes protease (PR), reverse transcriptase (RT), and integrase (IN), all of which provide essential enzymatic functions during viral replication.

A



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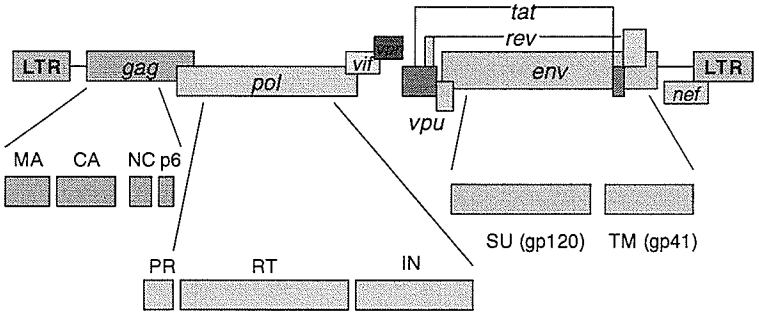


Figure1.1 HIV-1 virion structure and genome organization. A. Schematic representation of mature HIV-1 particle. Position of the proteins and genomic RNA are indicated. B. Organization of the HIV-1 genome and its structure and enzymatic gene products.

The six remaining genes, *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu*, are regulatory and accessory genes for proteins that control the ability of HIV to infect cells, produce new

copies of virus, or cause disease. For example, Tat is critical for HIV-1 transcription initiated from the long terminal repeat (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 as "accessory" or "auxiliary" proteins since that they are not absolutely required for virus replication *in vitro*. However, *in vivo*, these proteins indeed play important roles for efficient virus replication and disease progression. For instance, Nef appears to be necessary for efficient virus replication and stimulates virus infectivity. It also plays a role in down regulation of CD4 and major histocompatibility class I (MHC I) molecules from the cell surface. The *vpu*-encoded protein enhances the release of new virus particles from infected cells (Terwilliger et al., 1989) and promotes the degradation of CD4 through the host ubiquitin/proteasome pathway (Margottin et al., 1998). Vpr, another viral accessory protein, is incorporated efficiently into virions by a specific interaction with the p6 gag protein (Cohen et al., 1990; Kondo et al., 1995; Paxton et al., 1993; Yao et al., 1999) and efficiently arrests the cell cycle in G2 phase, and then induce apoptosis. Vpr could also weakly stimulate gene expression from the HIV LTR and might play a role in nuclear import of the viral preintegration complex (PIC) (Connor et al., 1995; Heinzinger et al., 1994; Nie et al., 1998; Vodicka et al., 1998). Vif is a basic protein which is incorporated into virions and is required in virus-producing cells during the late stages of infection to enhance viral infectivity (Kao et al., 2003; Strebel et al., 1987). 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, and Jurkat) are "permissive" for Vif mutants; virus produced from these cell lines is fully infectious regardless of the target

cell used (Freed, 2001). In contrast, other cell types (macrophages, primary human T cells and some restrictive T cell line) are "non-permissive" (Borman et al., 1995; Madani and Kabat, 2000). This cell-type specificity suggests that host factors play a role in Vif function. In non-permissive cells such as macrophages and primary human T cells, Vif functions to counteract an anti-retroviral cellular factor named APOBEC3G (Apolipoprotein B mRNA-editing Enzyme-Catalytic Polypeptide-like 3G) (Sheehy et al., 2002). The current mechanism proposed for protection of the virus by HIV-1 Vif is to induce APOBEC3G degradation through a ubiquitination-dependent proteasomal pathway (Liu et al., 2004).

1.2 HIV-1 replication cycle

HIV-1 infection begins with the binding of the virus to specific receptors (CD4) and co-receptors (CCR5 or CXCR4) on the cell surface, leading to their entry into the cell. Once in the cytosol, the genomic HIV-1 RNA is reverse-transcribed into a linear doubled-stranded DNA by the viral reverse transcriptase (RT). The viral DNA migrates to the nucleus wherein viral integrase (IN) catalyzes its integration into the cell genome. These two steps of the HIV-1 cycle take place inside large protein complexes termed the reverse transcription complex (RTC) and preintegration complex (PIC). Once integrated, the viral DNA is transcribed by the cellular machinery and messenger RNAs and progeny virion RNA are generated. Viral proteins and genomic RNA assemble at the cell periphery and immature virions are released by budding. Finally, the viral protease (PR) carries out cleavage of the precursor polypeptides including Gag, Gag-Pol to produce infectious viruses (Fig.1.2). Briefly, the whole life cycle of HIV-1 can be divided into

two distinct phases: "early" and "late". The early phase refers to the steps from susceptible 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. Next, the details of each replication step will be further described as follows:

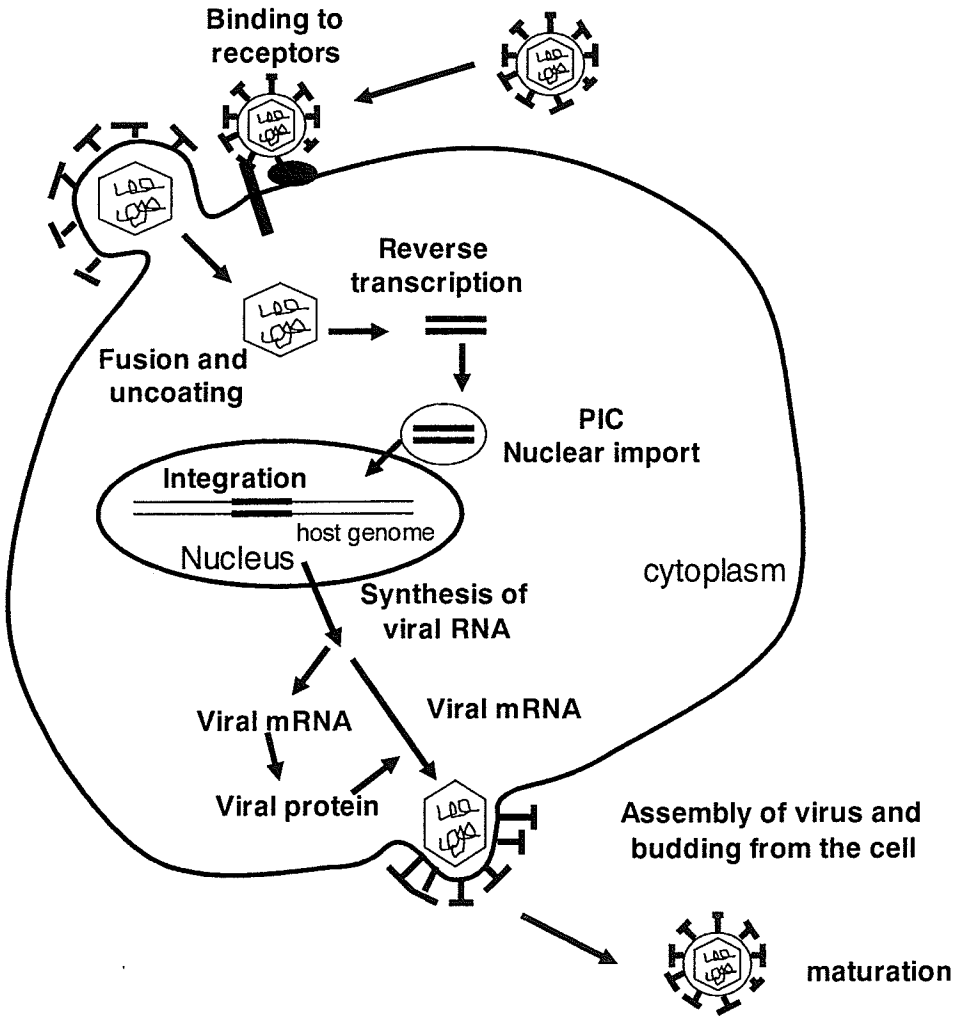


Figure1.2 Diagram of HIV-1 life cycle. Different steps in the cycle are shown starting from the entry of the viral particle into the cell until the budding of newly assembled virions.

1) HIV-1 Entry:

Once an infectious HIV-1 virion has entered the host body, it will specifically find and bind to the CD4 receptor expressed on the cell surface of macrophage, dendritic cell, or helper T lymphocyte. Virion attachment to the CD4+ target cell membrane is through the binding of the HIV-1 glycoprotein gp120 to the CD4 protein. The initial interaction of gp120 with CD4 is not sufficient for HIV-1 entry into the cell, but causes a conformational shift in the gp120 molecule to expose its chemokine co-receptor binding site. Depending on the viral tropism, cellular co- receptors CCR5 or CXCR4 are engaged which is determined by the V3 loop of gp120 (Chan and Kim, 1998; Wyatt and Sodroski, 1998). Once both the cell surface receptors are bound by gp120, the viral transmembrane protein gp41 changes conformation to facilitate membrane fusion with subsequent viral entry into the cell (Markosyan et al., 2003; Melikyan et al., 2000).

2) Uncoating and reverse transcription:

Once inside the cell, the virion core is uncoated, exposing the HIV nucleoprotein complex, consisting of genomic RNA, reverse transcriptase (RT), integrase, the matrix protein, Vpr and NC. 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 et al., 2000). RT first catalyzes the RNA-dependent DNA polymerization of the RNA genome into an RNA-DNA hybrid. The RNase H domain of RT then cleaves off the RNA, and RT subsequently becomes a DNA-dependent DNA

polymerase to generate the double stranded DNA viral genome. The fidelity of the reverse transcription is affected by the presence of cellular protein APOBEC3G (Goncalves et al., 1996; Mangeat et al., 2003; Mariani et al., 2003; Zhang 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 (Aiken, 1997; Aiken and Trono, 1995). In addition, the cellular protein cyclophilin A (CypA) was also found to enhance HIV infectivity during early post-entry events by counteracting the inhibitory activity of host restriction factor Ref1 and allowing reverse transcription to be completed (Towers et al., 2003).

3) HIV nuclear import:

HIV-1, as one of retroviruses, after penetrating into the host cells and completing reverse trancription, has to reach their sites of replication, the nucleus. Research has shown that HIV-1 cores use cellular microtubules to transport its genome toward the cell nucleus (McDonald et al., 2002) and during this time the viral PIC is formed. In addition to proviral DNA, RT and IN, the PIC includes other viral proteins such as MA , Vpr and NC, and cellular proteins such as the high mobility group protein HMGI (Y) , integrase interacter 1 (INI1), the barrier auto-integration factor (BAF) and the human lens epithelium-derived growth factor/transcription coactivator p75 (LEDGF/p75) (Farnet and Bushman, 1997; Llano et al., 2004b). However, the clear picture of the architecture of the PIC remains to be established. The HIV-1 cDNA, associated to viral and cellular proteins, has to cross the nuclear membrane to reach the host chromatin for its integration. However, the mechanism by which the PIC translocates into the nucleus is still not fully understood. It is recognized that HIV-1 PIC enters the nucleoplasm through

the nuclear pore complex (NPC) by active transport (Bukrinsky et al., 1992; Weinberg, 1991). 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 HIV-1 nuclear import (Bukrinsky et al., 1993; de Noronha et al., 2001; Gallay et al., 1997; Haffar et al., 2000; Heinzinger et al., 1994; Nie et al., 1998; Vodicka et al., 1998; Zennou et al., 2000). Moreover, further investigations for cellular proteins contained in the PIC are also urgently needed to elucidate their roles during viral nuclear import. And this will also provide us the opportunity to design inhibitors for blocking virus infection.

4) Integration:

Following viral nuclear import, the double-stranded cDNA genome is then integrated into a host chromosome of the infected cell, which can occur at many target sites within the host's genome. HIV integrase performs a number of catalytic steps for this viral integration, presumably with the help of additional host cellular enzymes for the integration and DNA repair. 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. Many more details about the HIV-1 integration reaction will be further discussed in part **1.5.1** of this chapter.

5) Viral gene expression:

The HIV-1 promoter is located in the 5'-long terminal repeat (LTR), and transcription of the integrated retroviral genes is performed by the host cell RNA polymerase II. During this process, the viral protein, Tat, plays a critical role for facilitating the transcription elongation processivity of the polymerase (Harrich and Hooker, 2002; Harrich et al., 1996). Transcription of the HIV provirus can be characterized by two phases. One is the early Tat-independent phase and another one is the late Tat-dependent phase. In the absence of Tat, a series of short transcripts are produced due to inefficient elongation by the recruited RNA polymerase II and the HIV promoter is strictly under the control of the local chromatin environment and cellular transcription. This process results in the synthesis of basal amounts of Tat proteins (Jordan et al., 2001; Kao et al., 1987). However, once Tat is present, RNA synthesis is greatly increased. Tat activates transcription through binding to TAR element of LTR and to other transcriptional activators of cellular origin (Harrich and Hooker, 2002; Harrich et al., 1996). 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 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 particle packaging and maturation:

The final step of the viral cycle, packaging of new HIV-1 virons, begins at the plasma membrane of the host cell. Viral transcripts bound by Rev and exported to the cytoplasm are translated into protein for the formation of infectious virion particles. The Env polyprotein (gp160) goes through the endoplasmic reticulum and is transported to the Golgi complex where it is cleaved by cellular protease and processed into the two HIV envelope glycoproteins gp41 and gp120. These two proteins are then transported to the plasma membrane of the host cell where gp41 anchors the gp120. The polyproteins Gag and Gag-Pol are also localized to the cell membrane along with the HIV-1 genomic RNA by the N-terminal matrix section of the polyprotein (Barreca et al., 2003; Derdowski et al., 2004; Sandefur et al., 2000). Once the subunits are assembled at the inner surface of the cell membrane, the viral particles begin to bud from the cell surface, coated with gp120 and gp41 and containing an unrefined virus interior. These noninfectious particles are then released and undergo a maturation process involving processing of Gag and Gag-Pol by HIV protease and assembly of the core particle. Protease digests the polyproteins into MA, CA, NC, p6, PR, RT, and IN (Quillent et al., 1996; Ross et al., 1991). This mature HIV virion is now ready to infect the next cell.

1.3 HIV-1 IN

HIV-1 IN, a 32-KD protein generated by protease-mediated cleavage of HIV-1 Gag-Pol polyprotein, catalyzes the integration of the reverse transcribed viral DNA into the host genome which is required for viral replication and chronic infection. IN carries out DNA integration in a two step reaction, called 3' processing and strand transfer (Fig.

1.5). The detailed integration reaction will be described in part 1.5.1 of this chapter. Given the fact that IN has no known cellular counterpart and that it is an essential viral enzyme for productive viral infection, isolation of specific inhibitors of this enzyme should provide novel anti-HIV therapeutic strategies (Neamati, 2001; Pani and Marongiu, 2000; Pommier et al., 2000). In contrast to RT and PR, the other two HIV-1 encoded enzymes currently used as targets in the combined therapy strategy, almost none of the few inhibitors of HIV-1 IN which have until now been described seem to behave as a potential therapeutic agent. This lack of IN inhibitors is partly due to the difficulties encountered in structural studies because of the low solubility of the whole enzyme and to insufficient information concerning the biochemical mechanism of proviral integration. Therefore, extensive efforts have been made to investigate the molecular basis of HIV-1 integration and to develop anti-IN inhibitors *in vivo*.

In vivo and *in vitro* complementation studies suggest that the functional HIV-1 IN is a multimer (Fletcher et al., 1997; Kalpana et al., 1999). Moreover, oligomers of IN are present in virions (Petit et al., 1999). However, the number of monomers and the spatial organization of the active oligomeric form of IN complexed with DNA substrate is not yet clear. Biochemical and structural considerations suggest that the minimal functional IN oligomer is at least a tetramer (Esposito and Craigie, 1999). The presence or the absence of divalent cations or detergents during the purification process of IN would explain why IN exists in monomers, dimers, tetramers, and high-order multimers *in vitro*.

1.4 Structures of HIV-1 IN

Protease digestion and functional complementation studies show that the full length HIV-1 IN (288 amino acids) contains three different domains (Fig.1.3): the N-terminal

domain (1-50), the catalytic core domain (51-212), and the C-terminal domain (213-288). The structure of each of these separate domains has been determined by X-ray diffraction or by solution NMR. Structures also exist for the core domain plus N-terminal domain (Wang et al., 2001) and for core domain plus the C-terminal domain (Chen et al., 2000). However, the complete structure of HIV-1 IN or its complex with DNA substrate is still unknown because of its low solubility. All three domains of HIV-1 IN are required for efficient integration activity.

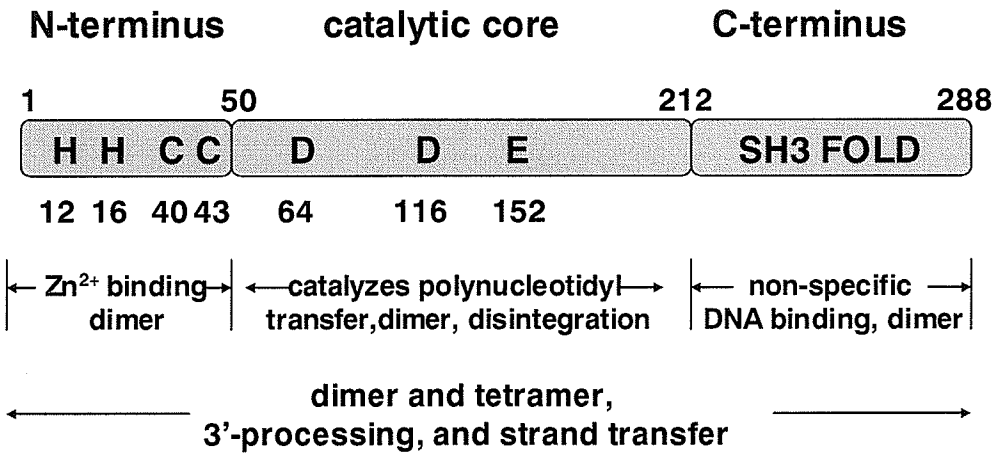


Figure1.3 Structural and functional domains of HIV-1 IN. IN is a three domain multimeric protein, that contains conserved amino acid sequence motifs in both the N-terminus (HHCC) and core domain (D,D-35-E). The function of each domain is indicated.

N-terminal domain

The N-terminal domain of HIV-1 IN contains a highly conserved HHCC (H12, H16, C40, C43) motif (Fig1.3), which binds one equivalent of Zn^{2+} for stabilizing the interaction between the helices and overall IN structure, as well as promoting the formation of higher-order IN multimers (Zheng 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). This region is also involved in protein-protein interactions and contributes to the specific recognition of viral DNA ends. The structure of the N-terminal domain of HIV-1 IN has been solved by NMR spectroscopy (Cai et al., 1997) and shows that N-terminal domain is a highly helical structure, 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 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.

Catalytic core domain

The catalytic core domain (CCD) of HIV-1 IN contains 3 highly conserved residues, Asp^{64} (D), Asp^{116} (D), and Glu^{152} (E) which are commonly found in polynucleotidyl transferases. Any mutation of these residues usually abolishes all catalytic activities of these proteins, and they are therefore thought to be essential components of the IN active site, termed as the catalytic D, D-35-E motif (Fig.1.3). 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 (Bushman et al., 1993; Engelman et al., 1993; Kulkosky et al., 1995; Mazumder et al., 1994; Schauer and Billich, 1992; Vink et al., 1993). The crystallization of the core domain was initially impeded by its poor solubility. Fortunately, later studies showed that an IN mutant F185K, generated by the systematic replacement of the hydrophobic residues, improved solubility and had as much activity for the disintegration reaction as the wild type domain

(Jenkins et al., 1995). After that, X-ray diffraction analysis shows the catalytic core domain of IN consists of a central five-stranded β sheet with six surrounding α helices (Fig.1.4). Examination of the crystal structure displays the contact between CCD monomers which suggests a dimeric model for functional IN. In this contact, the two monomers are related by a dyad axis, with a large, solvent-excluded surface. The dimer is stabilized by salt bridges and hydrogen bonds involving β strand 3 and α-helices 1, 3, 5, and 6.

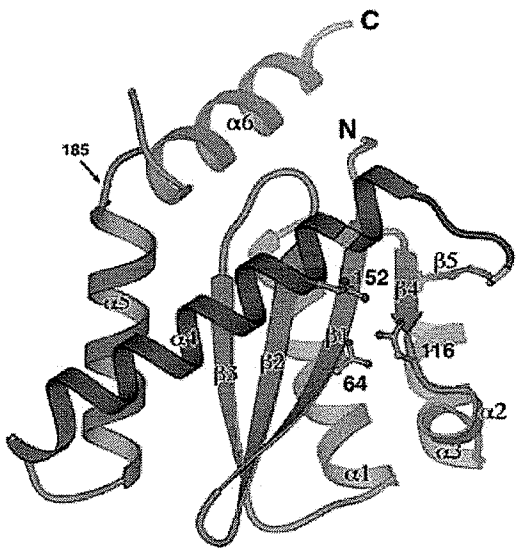


Figure1.4 The HIV-1 IN catalytic core domain. β strands and α helices are marked. Three residues of the D,D-35-E motif, Asp^{64}, Asp^{116}, and Glu^{152} are shown as ball-and-stick models.(from J. Greenwald et al.,1999)

C-terminal domain

The C-terminal domain is the least conserved of the three domains and binds DNA non-specifically. Deletion of this domain will abolish 3'-end processing and strand transfer activities. Two NMR studies on solutions of domains consisting of residues 219-270 showed five strands arranged antiparallel to form a β barrel which adopts an SH3-

like fold (Eijkelenboom et al., 1999; Lodi et al., 1995; Vink et al., 1993). SH3 domains are involved in protein-protein interactions. In particular, they may provide connections to small GTP-binding proteins. Another role that has been proposed for SH3 domains is the ability to interact with proteins of the cytoskeleton, thus trigging changes in cell structure. In both cases, the domains dimerize to form a symmetrical dimer. The crystal structure of the two domains of HIV-1 IN, residues 52-288 has also been determined by X-ray crystallography (Chen et al., 2000). Two C-terminal domains are related to each other by 90° rotation relative to their two- fold axis. Within the dimer, only the catalytic core domains form the dimer interface, and the C-terminal domains are located 55 Å apart. A 26-aa α-helix, α6, links the C-terminal domain to the catalytic core.

1.5 Functions of HIV-1 IN

As one of the three viral encoded enzymes, HIV-1 IN plays a major role in catalyzing the integration of proviral DNA into the infected cell genome. Moreover, in addition to catalyzing the integration process, HIV-1 IN has also been suggested to play other important roles during viral replication including reverse transcription and HIV-1 nuclear import. In this part, firstly, the discussion will be focused on the IN-performed integration reaction in which other cellular partners involved in this step are also described. Then the effects of HIV-1 IN on reverse transcription and nuclear import will be discussed.

1.5.1 Integration reaction

HIV-1 IN catalyzes DNA integration in two steps: 3'-end processing and 3'-end joining or strand transfer (Fig.1.5), which IN alone can carry out.

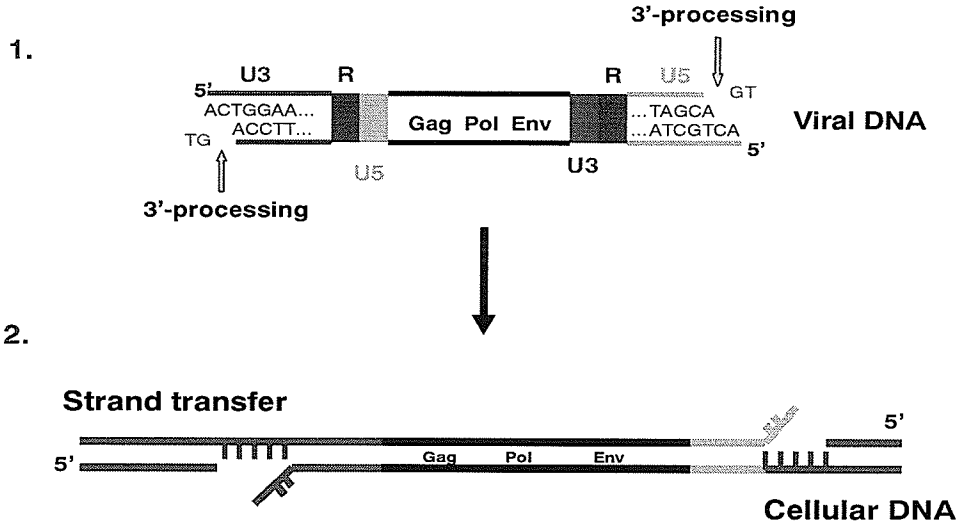


Figure 1.5 Diagram of the integration reaction. Catalytic steps involved in the insertion of viral DNA into the human genome are indicated as 1 and 2.

1) The first step in the integration reaction is "3'-processing" which happens in the cytoplasm within the PIC. In this processing reaction, IN specifically recognizes the sequence-specific 3' LTR on the linear double-stranded viral cDNA and then removes the terminal GT dinucleotide from each of the viral DNA ends, leaving a recessed CA with a free 3' OH group and an overhanging 5'-AC on the complementary strand.

2) The second step, termed strand transfer reaction, happens in the nucleus. The IN protein joins the previously processed 3' ends to the 5' ends of strands of target DNA at the site of integration. The 5' ends are produced by IN-catalyzed staggered cuts. The unpaired nucleotides at the 5'-ends of the viral DNA are then removed and the gaps

between the viral and the target DNA are repaired, probably by host-cell DNA repair enzymes (Chow et al., 1992).

IN also carries out a third reaction, called the disintegration reaction, which is the reverse of the strand transfer reaction in which a substrate that mimics one end of the viral DNA joined to the target DNA is cleaved into its viral and target DNA parts (Chow et al., 1992).

In vitro analysis has shown that only two elements are necessary for integration: the HIV-1 IN and the *cis*-acting DNA sequences at the end of the proviral DNA LTR. Although purified recombinant HIV-1 IN performs all the steps required for end processing and strand transfer on model DNA substrates *in vitro*, such reactions differ from authentic integration because coordinate joining of the two viral ends remains inefficient. *In vivo*, the enzyme may attain the expected efficiency by interacting with viral or cellular proteins present in the PIC which can efficiently facilitate the integration.

1.5.2 Cellular factors interacting with HIV-1 IN

In addition to the key viral protein of IN for HIV-1 integration, a variety of cellular proteins have been demonstrated to be involved in establishing the integrated provirus in the infected cells. Examples include the barrier-to-autointegration factor (BAF) (Lee and Craigie, 1998; Lewis and Emerman, 1994), high-mobility group protein A1 (HMGA1) (Farnet and Bushman, 1997), integrase interactor 1(INI-1) (Kalpana et al., 1994; Young, 2001), DNA-PK (Daniel et al., 1999), DNA repair protein hRAD18 (Mulder et al., 2002), a cellular acetyltransferase p300 (Cereseto et al., 2005), and lens epithelium-derived growth factor/p75 (LEDGF/p75) (Busschots et al., 2005). Since INI1 and LEDGF/p75

can directly interact with HIV-1 IN and have been studied well in detail as the cofactors of retroviral integration, here, we just focus our interest on them.

INI1: INI1, identified by the yeast "two-hybrid" system, is one of the proteins that directly interact with IN and is shown to stimulate the integration reaction *in vitro* (Kalpana et al., 1994). INI1 is a homologue of yeast transcription factor SNF5 and is a component of the ATP-dependent chromatin-remodeling mammalian SWI/SNF complex (Wang et al., 1996). It is involved in chromatin remodelling during gene expression. As retroviral integration seems to occur preferentially in highly transcribed loci(Scherdin et al., 1990), it is tempting to speculate that the role of INI1 in the retroviral infection cycle is to target the PIC by binding IN to accessible chromatin regions. INI1 is a 385-amino-acid protein and contains 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 et al., 1998). Morozov et al. also demonstrated that INI1 is incorporated into virions and the incorporation of INI1 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 et al., 2001). Moreover, a fragment of INI1 (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 et al., 2004). Therefore, INI1 may play a role during the post-integration steps of HIV-1 replication. Recently, Ariumi et al. also demonstrates

that while INI1 is dispensable for HIV-1 transduction, it can facilitate HIV-1 transcription by enhancing Tat function. INI1 bound to Tat and both the repeat (Rpt) 1 and Rpt 2 domains of INI1 were required for efficient activation of Tat-mediated transcription. These results suggest that the incoming PICs might recruit INI1 to facilitate proviral transcription (Ariumi et al., 2006).

LEDGF/p75: LEDGFp75 is a novel cellular protein which directly interacts with HIV-1 IN and targets HIV-1 DNA integration. It is predominantly localized in the nucleus, where it is associated with the chromosomes (Nishizawa et al., 2001). Normally, LEDGF/p75 functions as a survival factor and a transcriptional co-activator in the cell. 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 et al., 2003; Emiliani et al., 2005; Turlure et al., 2004). Recombinant LEDGF/p75 protein effectively promotes HIV-1 IN strand transfer activity *in vitro*.

LEDGF/p75 contains 530 amino acids and has several functional regions. Of note, a 92 amino acid domain that contains PWWP (Pro-Trp-Trp-Pro) is present in the N-terminus. This functions as a protein-protein interaction domain and/or DNA-binding domain (Qiu et al., 2002; Stec et al., 2000). Moreover, a functional NLS (^{146}RRGRKRKAEKQ^{156}) was also found in this region by deletion mapping and site-directed mutagenesis (Maertens et al., 2004; Vanegas et al., 2005). However, the conserved IN-binding domain (IBD) of 80 amino acids (residues 347-429) was mapped to the C-terminus (Cherepanov et al., 2004). The structure of the IBD has been resolved by NMR (Cherepanov et al., 2005) showing that it is a compact right-handed bundle

composed of five α helices. Also, in 2006, the domains responsible for the chromatin tethering of LEDGF/p75 were characterized. Immuno-localization analyses revealed that an N-terminal PWWP domain and its beta-barrel substructure are needed for binding to chromatin (Llano et al., 2006b). Moreover, LEDGF/p75-binding site on IN was also studied. 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 et al., 2003). Two regions within the central core domain of HIV-1 IN have also been characterized to interact with LEDGF/p75. The first region centers around residues W131 and W132 while the second extends from I161 through E170 (Busschots et al., 2007). For the different IN mutants, the interaction with LEDGF/p75 and the enzymatic activities were determined. IN(W131A), IN(I161A), IN(R166A), IN(Q168A) and IN(E170A) are impaired for interaction with LEDGF/p75, but retain 3' processing and strand transfer activities. Due to impaired integration, an HIV-1 strain containing the W131A mutation in IN displays reduced replication capacity, whereas virus carrying IN(Q168A) is replication defective (Busschots et al., 2007).

In addition to the *in vitro* interaction between LEDGF/p75 and IN, the functional roles of LEDGF/p75 in HIV-1 replication have also been studied. 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 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 et al., 2004). Therefore, a possible role of LEDGF/p75 in nuclear import or, alternatively, in chromosomal tethering was initially proposed

(Maertens 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 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 (Emiliani et al., 2005; Llano et al., 2004a). Recently, in fluorescent correlation spectroscopy experiments, LEDGF/p75 was found to stimulate the binding of HIV-1 IN to DNA (Busschots 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, Llano et al found that LEDGF/p75 is an essential HIV integration cofactor. The mechanism requires both linkages of a molecular tether that p75 forms between IN and chromatin (Llano et al., 2006a). Ciuffi et al also demonstrated that LEDGF/p75 may affect the choice of target sites for HIV-1 integration in cells (Ciuffi et al., 2005). In 2006, Vandekerckhove et al. found that transient and stable knockdown of LEDGF/p75 resulted in a three- to fivefold inhibition and a two- to fourfold reduction of HIV-1 replication respectively indicating that the extent of LEDGF/p75 knockdown closely correlated with the reduction of HIV-1 replication (Vandekerckhove et al., 2006). Furthermore, two different overexpressed fragments containing the IN binding domain (IBD) of LEDGF/p75 have been shown to inhibit HIV-1 replication. Quantitative PCR pinpointed the block to the integration step, whereas nuclear import was not affected. Competition of the IBD proteins with endogenous

LEDGF/p75 for binding to integrase led to a potent defect in HIV-1 replication (De Rijck et al., 2006).

1.5.3 Characterization of class I and class II IN mutants.

Replication-defective IN mutants of HIV-1 can be grouped into two phenotypic classes: class I and class II.

Class I IN mutants are specifically blocked at the integration step and are typified by changes in the D,D-35-E motif that constitutes the enzyme active site. In contrast, class II mutants cause pleiotropic defects at multiple stages of viral replication other than integration, including early and postintegration steps of the virus life cycle. For examples, some IN mutant viruses are shown to be defective at postintegration steps such as virion assembly, release, maturation, and protein composition (Ansari-Lari et al., 1995; Bukovsky and Gottlinger, 1996; Engelman et al., 1995; Engelman et al., 1997; Quillent et al., 1996; Shin et al., 1994). Some other defective IN mutants viruses are impaired in early steps of the virus life cycle, such as uncoating, viral DNA synthesis and nuclear import of PIC (Bouyac-Bertoia et al., 2001; Cannon et al., 1996; Engelman et al., 1995; Engelman et al., 1997; Gallay et al., 1997; Leavitt et al., 1996; Masuda et al., 1995; Wu et al., 1999). 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 causes each defect. For example, besides *in vitro* integration assay, the Alu-PCR and complementation assay are used to detect integration

in vivo. (Chun et al., 1997). For reverse transcription, 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 et al., 2002b). Detection of 2 LTR circles is the most widely used assay for monitoring nuclear translocation of the PIC (Lewis, 1992).

1.5.4 Contribution of HIV-1 IN on reverse transcription.

Although reverse transcription can be catalyzed by RT alone *in vitro*, the process is more complex *in vivo*. In infected cells, in addition to several viral factors such as MA, NC, Nef, and Vif, IN is also involved in this process (Tasara 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 et al., 1995; Engelman et al., 1997; Liu et al., 1999; Masuda et al., 1995; Wu 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 et al., 1986; Tasara et al., 2001; Wu et al., 1999). 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 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 et al., 2004; Zhu et al., 2004).

1.5.5 Contribution of HIV-1 IN on PIC nuclear import.

Besides its critical role for catalyzing the integration of HIV-1 cDNA into the host genome, IN can also contribute to the viral PIC nuclear import. In 1997, Gallay *et al.* first reported that HIV-1 IN was able to localize in the nucleus by recognizing importin-α (one cellular karyophilic protein) via its bipartite NLS ^{186}KRK^{188} and ^{211}KELQKQITK^{219} since mutants K186Q and Q214/216L in these regions lost nuclear localization capacity and their ability to bind to importin α *in vitro* (Gallay et al., 1997). Consistently, the karyophilic feature of IN was further confirmed by different groups using various IN-fusion proteins. However, other studies on the subcellular localization of IN could not prove the importance of ^{186}KRK^{188} and ^{211}KELQKQITK^{219} for protein nuclear localization and/or their roles in viral nuclear import; rather they appear to contribute to reverse transcription and/or integration (Bouyac-Bertoia et al., 2001; Lu et al., 2004; Petit et al., 2000; Tsurutani et al., 2000). Moreover, although Bouyac-Bertoia *et al.* reported a nonclassical NLS within the catalytic core domain of IN (Bouyac-Bertoia et al., 2001), later reports were unable to confirm this observation (Dvorin et al., 2002; Limon et al., 2002a). Interestingly, in 2005, Ao et al. demonstrated that replacing lysine residues with alanine in two highly conserved tri-lysine regions, which are located within previously described Region C (^{235}W**K**GPA**K**LLW**K**GEGAVV^{250}) and sequence Q

(^{211}**K**ELQ**K**QIT**K**^{219}) in the C-terminal domain of HIV-1 IN, impaired protein nuclear accumulation (Ao et al., 2005).

In 2001, the residues V165 and R166 in HIV-1 IN were shown to be critical for its NLS function (Bouyac-Bertoia et al., 2001). But reassessment 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 et al., 2002; Limon et al., 2002b). Recently, mutants V165A and R166A were identified to be defective for binding to cellular protein LEDGF/p75 by His6-tag pull down assay (Cherepanov 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 et al., 2005).

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 (Armon-Omer et al., 2004; Fassati et al., 2003; Gallay et al., 1997), while Depienne *et al.* revealed that IN nuclear accumulation *in vitro* neither involved importin α, β1, and β2-mediated pathways, nor GTP hydrolysis (Depienne et al., 2001). Moreover, other studies have implicated nuclear translocation of IN to nucleus by its interaction with a cellular component LEDGF/p75 (Cherepanov et al., 2003; Maertens 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 (Emiliani

et al., 2005; Llano et al., 2004a; Llano et al., 2004b). Most recently, another cellular protein importin 7 has been shown to interact with IN and have positive impact on viral replication (Ao et al., 2007).

Taken together, it appears that 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.

1.6 Functional characterization of HIV-1 IN in yeast.

HIV-1 IN plays multiple roles during the early phase of HIV-1 replication, including reverse transcription, viral DNA nuclear import and integration (Ao et al., 2005; Bukovsky and Gottlinger, 1996; Engelman et al., 1995; Gallay et al., 1997; Ikeda et al., 2004; Nakamura et al., 1997; Wu et al., 1999). Therefore, it is difficult to specifically assess one particular function of IN during viral replication due to the fact that most IN mutants exhibit pleiotropic phenotypes. To facilitate the integration study without being affected by other functions of IN, various *in vitro* assays and *in vivo* analyses, including yeast IN expression system have been developed to assess different activities of HIV-1 IN during integration. In particular, previous studies have shown that the expression of functional HIV-1 IN in some *Saccharomyces cerevisiae* yeast strains, such as the protease deficient haploid JSC 302, RAD52 deficient haploid W839-5C, and diploid AB2 leads to the emergence of a lethal phenotype that may be related to the HIV-1 integration reaction (Caumont et al., 1996; Parissi et al., 2000a; Parissi et al., 2000b).

HIV-1 IN behaves in the yeast cell context in a way similar to its natural activity in the human infected cell and the yeast lethal phenotype system is a powerful model to study the retroviral integration mechanism *in vivo.* Moreover, this yeast system also allows us to characterize the IN activity in a eukaryotic cellular context and to select IN mutations affecting both the lethal phenotype and in vitro IN activities. More specifically, it is also used to identify the cellular partners interacting with IN which are involved in the proviral DNA integration. Therefore, the yeast model is a useful tool to investigate some of IN activities during the integration process and the information derived from this system may provide great insights into the mechanisms how HIV-1 IN interacts with cellular machinery.

HIV-1 IN-induced lethal effect in yeast seems to be associated with yeast genomic DNA damage produced by the non-sequence-specific endonucleolytic activity carried by IN since an IN catalytic mutant (D116A) was unable to induce the lethality in yeast (Parissi et al., 2003; Parissi et al., 2000b). This non-sequence-specific endonuclease activity was further characterized by in vitro assays showing that even in the absence of viral long terminal repeat (LTR) sequences, IN is known to non-specifically cleave the DNA substrate and to produce DNA breaks indicating that this reaction could be independent of the processed viral ends (Sherman and Fyfe, 1990).

The IN endonucleolytic activity participates actively in retroviral integration since breaks of the host genome are crucial steps for viral DNA integration. This endonucleolytic activity inducing yeast genomic DNA damage was also supported by the fact that disruption of the *rad 52* gene, which is involved in the repair of double-strand DNA breaks, strongly increased the deleterious effects of the retroviral enzyme in W839-

5C compared with the wild type RAD52 strain W303-1A (isogenic to W839-5C except for the *rad* 52 gene in its wild-type form) (Caumont et al., 1996).

However, in 2004, Calmels *et al.* revealed that another specific mutation targeting amino acid E152, one of the three crucial triad residues (D64D116(35)E152) of IN did not disrupt IN's lethal activity in yeast (Calmels et al., 2004). Thus, the mechanism underlying the IN induced yeast lethal phenotype is still not fully understood and other un-characterized activities of HIV-1 IN around the integration step, such as chromatin binding ability, might play an important role for its lethal activity in *S. cerevisiae.*

Furthermore, the following studies that use this yeast lethal phenotype system, revealed that IN was unable to mediate its lethal activity in yeast cells when a SNF5 gene, which encodes a component of the SWI/SNF chromatin remodeling complex, was disrupted, suggesting a role of SNF5 for IN-induced lethal phenotype in yeast (Parissi et al., 2000a). Given the homology between SNF5 and its human counterpart IN interactor 1 (INI1) (Kalpana et al., 1994), this factor might also be important for IN activity in infected cells. Moreover, new potential factors like chaperonin hHSP60 are also necessary for the activity of IN in yeast (Parissi et al., 2001).

1.7 Objectives of this project

HIV-1 belongs to the *lentiviridae* genus of retroviruses and its replication is dependent on the integration of the reverse-transcribed viral genome into the host chromosome. The HIV-1 IN is the key viral enzyme required for this integration step. This enzyme has also been demonstrated to play other important roles for different early steps prior to integration, such as viral reverse transcription and viral DNA nuclear import (Ao et al., 2005; Bukovsky and Gottlinger, 1996; Engelman et al., 1995; Gallay et

al., 1997; Ikeda et al., 2004; Nakamura et al., 1997; Wu et al., 1999). In this study, we just focused our interest on viral integration step. Although previous data have shown that HIV-1 IN catalyzes the integration accurately *in vitro*, a variety of other questions still remain unclear. For example, is IN alone sufficient for integration *in vivo*; how does it interact with the cellular machinery; and what critical amino acids besides D,D,(35)E catalytic motif of IN are required for the integration? In this study, we would like to further study the mechanisms underlying its action during viral integration. Interestingly, previous studies have shown that expression of HIV-1 IN in some yeast strains lead to the emergence of a lethal phenotype which is closely related to the HIV-1 integration reaction(Caumont et al., 1996; Parissi et al., 2003). This yeast lethal phenotype system provides us an ideal model to study the integration reaction without being influenced by other replication steps prior to integration such as reverse transcription and nuclear import. Therefore, in this study, we first would like to perform mutagenic analysis to investigate how different IN mutants affect their lethal activity in yeast and then based on the results from yeast we would like to further study the effect of different IN mutants on viral replication. Finally, we would try to clarify the mechanism involved in yeast lethality and viral replication. The specific objectives of this project are to:

1. Establish the HIV-1 IN-induced lethal phenotype system in certain yeast strains including haploid HP16 and diploid BY4743.

2. Do further mutagenic analysis to determine which amino acids and/or regions of HIV-1 IN are important for this lethal effect in yeast.

3. Further investigate the effect of different IN mutants on viral replication by using one single cycle replication system.

4. Perform functional and biochemical analyses to explore the molecular mechanism of HIV-1 IN-induced yeast lethality and viral replication.

CHAPTER 2 MATERIALS AND METHODS

2.1 Yeast strains, culture media, and growth conditions

To investigate the effect of HIV-1 IN in *Saccharomyces cerevisiae*, 3 different yeast strains were used. They were: the protease-deficient haploid HP16 strain (*MAT ura3-52; his3Δ1; leu2; trp1Δ63; prb1-1122; pep4-3 prc1-407*) which had been described previously (Park et al., 1993; Yao et al., 2002); diploid BY4743 ($rad52^+$) strain(*MATa/α;his3Δ1/his3Δ1;leu2Δ0/leu2Δ0;lys2Δ0/LYS2;MET15/met15Δ0;ura3Δ0/ura 3Δ0;YML032c::kanMX4/YML032c)* and diploid BY4743($rad52^-$) strain (*MATa/α;his3Δ1/his3Δ1;leu2Δ0/leu2Δ0;lys2Δ0/LYS2;MET15/met15Δ0;ura3Δ0/ura3Δ0; YML032c::kanMX4/YML032c::kanMX*4). The only difference between the latter two strains is that BY4743 ($rad52^-$) strain does not contain the RAD52 encoding gene but does the BY4743 ($rad52^+$) strain. Both of the latter two strains were purchased from EUROSCARF collection center (Frankfort, Germany).

All three yeast strains were able to grow well in yeast complete media YPD (1% yeast extract, 2% peptone, 2% glucose, with or without 2% agar). However, according to different yeast strains, different selective culture media were employed for yeast cultivation. For yeast strain HP16, yeast liquid selective media lacking tryptophan (0.67% yeast nitrogen base without amino acids, 0.5% casamino acids (tryptophan$^-$(Trp$^-$), uracil$^-$ (Ura$^-$), adenine$^-$(Ade$^-$)), 20mg/l uracil, 20mg/l adenine, 2% galactose or raffinose) were employed. In contrast, for diploid BY4743($rad52^+$) and diploid BY4743($rad52^-$) strains, liquid selective media lacking histidine (0.67% yeast nitrogen base without amino acids, 0.5% casamino acids (histidine$^-$(His$^-$), uracil$^-$ (Ura$^-$)) , 20mg/l uracil , 2% galactose or

raffinose) were used. The corresponding solid selective media were obtained by supplementing liquid media with 2% agar. Liquid cultures were performed in flasks filled to one fifth of their capacity and receiving vigorous agitation. All yeast strains were grown at 30 °C.

2.2 High efficiency transformation of yeast

To study the HIV-1 IN-induced lethal phenotype in each yeast strain, the wild type and/or mutant IN expressing plasmids were transformed into yeast cells using the lithium acetate method as described before (Gietz et al., 1992). Briefly, 30µl of each yeast sample taken from frozen stocks was inoculated into 5 ml complete YPD media and incubated with shaking overnight at 30°C. Overnight culture was counted and inoculated into 50 ml of YPD media to make a cell density of $5x10^6$/ml. The inoculum was then incubated at 30°C with shaking at 200 rpm until it reached 2 $x10^7$ cells /ml density. This culture would give sufficient cells for 10 transformations. Next, the culture was centrifuged at 3000 x *g* for 5 minutes in a sterile 50-ml centrifuge tube. The cells were resuspended in 25 ml of sterile H_2O, and centrifuged again. Pouring off the H_2O, the cells were then re-suspended in 1.0 ml of 100 mM lithium acetate (LiAc), and the suspension was transferred into a sterile 1.5 ml Eppendorf tube. The cells were centrifuged at 13,000 rpm for 5 seconds and the LiAc was removed. Yeast cells were then resuspended with 100 mM LiAc to a final volume of 500µl ($2x10^9$ cells /ml), which usually required about 400µl of 100 mM LiAc. The cell suspension was votexed and 50µl of samples was added into labelled 1.5ml eppendorf tubes. The cells were spun down and LiAc was removed. Meanwhile, 1.0 ml sample of single-stranded carrier DNA was boiled for 5 minutes and quickly chilled in ice water. The ingredients consisting of the basic "transformation mix" were then

carefully added in the following order: 240μl of PEG (50%W/V), 36μl of 1.0 M LiAc, 25μl of single-stranded carrier DNA (2.0mg/ml), 50μl of H_2O and plasmid DNA (0.1-5μg). Each tube was votexed vigorously until the cell pellet was completely mixed. The transformation mix was then incubated for 30 minutes at 30°C and heat-shocked for 20-25 minutes at 42°C. Yeast cells were centrifuged at 8000 x *g* for 15 seconds and the transformation mix was removed. Then, 1.0 ml of sterile H_2O was added into each tube and the cell pellet was resuspended by pipetting up and down gently. Finally, 100μl of the transformation mix was plated onto each corresponding selective media agar plate and incubated for 3 to 5 days at 30°C for selecting positive clones.

2.3 Cell lines and transfections

Human embryonic kidney 293T and the African green monkey kidney COS-7 cell lines were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin and streptomycin. Human $CD4^+$ C8166 T-lymphoid cells were maintained in RPMI-1640 medium supplemented with 10% FCS and 1% penicillin and streptomycin. 293T cells were used for HIV-1 IN chromatin binding assay and single cycle replicating virus production. COS-7 cells were employed for immunofluorescence experiment. Human $CD4^+$ C8166 T cells were used for HIV-1 infection assay. The detailed experimental procedures will be further described in the following section.

DNA transfection in 293T and COS-7 cells were performed with standard calcium phosphate DNA precipitation method, as previously described (Yao et al., 1995). Briefly, 60-80% confluent cells were reached by splitting the day prior to transfection. Plasmid DNA (1-8μg as required), sterile ddH_2O and 2M $CaCl_2$ were mixed together and

transferred into 2X HBS buffer(HEPES-Buffered Saline) (50mM HEPES (pH 7.1), 280mM NaCl ,1.5mM Na_2HPO_4). After sitting for 15-30 minutes at room temperature, the calcium phosphate precipitates were mixed and added to a corner of the cell culture plate. After 40-48 hours incubation at 37°C with 5% CO_2, with changing the medium at 8 - 12 hours post-transinfection, the cells were harvested for each experimental purpose. Particularly, for immunofluorescence experiment, COS-7 cells were grown on glass coverslip (12 mm^2) in 24-well plate instead of tissue culture plates before performing DNA transfection.

2.4 Plasmids and antibodies

To test the lethal activity of HIV-1 wild type and/or mutant IN in each yeast strain, different IN-expressing plasmids were constructed. For yeast strain HP16, one HIV-1 IN yeast expression plasmid (p424Gal1-IN) was generated by inserting a PCR-generated *BamHI-PstI* fragment containing an IN sequence into a high copy yeast expression plasmid vector, p424Gal1 (Mumberg et al., 1994), which harbours a galactose-inducible Gal1 promoter and a tryptophan selection marker. To generate different p424Gal1-IN mutants expression plasmids, each IN mutant cDNA was generated by a two-step mutagenic polymerase chain reaction (PCR)-based method (Yao et al., 1995) with each corresponding oligonucleotide primers containing the desired mutations. Amplified IN cDNA harbouring specific mutation was then cloned into the p424Gal1 vector at *BamHI/PstI* sites. All IN mutants were sequenced to confirm the presence of mutations (ABI 3100 sequencer Prism Big Dye terminator cycle sequencing ready reaction kit; Applied Biosystem). For yeast strains BY4743 (*rad52*$^+$) and BY4743 (*rad52*$^-$), another HIV-1 IN yeast expression plasmid (p423Gal1-IN) was also constructed by using

different restriction enzyme sites which was a similar method as described above. The only difference between yeast expressing vectors p424Gal1and p423Gal1 is that p423Gal1 contains a histidine selection marker instead of the tryptophan selection marker carried by p424Gal1. Some IN mutants were also introduced into this plasmid by using the same procedure as indicated above.

To specifically investigate the effect of different IN mutants on early steps of viral replication, we used a RT/IN/Env gene-deleted HIV-1 provirus NL4.3Luc/ΔBgl/ΔRI, in which the *nef* gene was replaced by a firefly luciferase gene (Ao et al., 2005). To functionally complement RT/IN defects of NL4.3Luc/ΔBgl/ΔRI provirus , a CMV-Vpr-RT-IN fusion protein expressor was used in this study in which different mutants including V165A, A179P, and KR186,7AA or D64E were introduced into CMV-Vpr-RT-IN expressor by PCR-based method as described before (Ao et al., 2004). Co-transfection of NL4.3Luc/ΔBgl/ΔRI, CMV-Vpr-RT-IN and a vesicular stomatitis virus G (VSV-G) glycoprotein expressor in 293T cells results in the production of VSV-G pseudotyped HIV-1 particles that can undergo single cycle replication in C8166 cells. 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.

To test the association between HIV-1 IN and cellular chromatin, different hemagglutinin (HA)-tagged IN expression plasmids (SVCMV-HA-INwt/mut) were constructed by fusing IN cDNA to 3' end of cDNA to the HA sequence (5'-ATGGCTTCTAGCTATCCTTATGACGTGCCTGACTATGCCAGC-3'). For the

intracellular localization experiments, SVCMV-INwt/mut-YFP were constructed as described previously (Ao et al., 2005) .

Antibodies used in the immunofluorescence assay, immunoprecipitation or western blot were as follows: The HIV-1 positive human serum 162 was previously described (Yao et al., 1998). The mouse monoclonal antibody against yeast β-actin (ab8224) was purchased from Abcam Inc. The rabbit anti-YFP and anti-HA antibodies were purchased from Molecular Probes Inc. Anti-IN antibodies were kindly provided by Dr. D. Grandgenett through AIDS Research Reference Reagent Program, Division of AIDS, NIAID, NIH.

2.5 Evaluation of the lethal phenotype induced by HIV-1 wild type or mutant IN in yeast strains.

The experimental procedures to evaluate the growth arrest activity in yeast were described previously (Yao et al., 2002). Briefly, different yeast cells transformed with wild type or mutant IN plasmids (p424-Gal1-$IN_{wt/mut}$ and/or p423-Gal1-$IN_{wt/mut}$) were first grown in an appropriate IN non-inducible selective media (2% raffinose) for 2 days. Then, equal amounts of transformed yeast cells (~20000 cells) were inoculated into appropriate IN non-inducible (2% raffinose) or IN-inducible (2% galactose) selective media for liquid assay. After 24hr shaking cultivation at 30°C, yeast growth was monitored by measuring each yeast cell culture density by spectrophotometric analysis at 600nm (*A*600). Meanwhile, equal amounts of transformed yeast cells were serially diluted and spotted onto either an IN non-inducible or inducible agar plate for "drop test". After incubation for 3 to 5 days at 30°C, yeast colony numbers were recorded by photograph.

2.6 Detection of HIV-1 IN expression in yeast

To detect IN expression in yeast, the equal amounts of yeast cells transformed with IN-expressing or control plasmid were grown in IN inducible selective media (2% galactose) for 6 hours, then yeast cells were pelleted by centrifugation at 3000 x *g* for 15 min and then lysed in RIPA lysis buffer (150 mM Tris–HCl, (pH 8.0), 150 mM NaCl, 0.5% DOC, 0.1% (w/v) SDS, 1% (v/v) NP-40) using four cycles of vortex in the presence of glass beads for 1 min on ice. Supernatant was collected and IN protein was immunoprecipitated with anti-HIV antibodies for 3 hours at 4°C. After that, appropriate amount of proteinA sepharose beads were added into the cell lysate and incubated for another 2 hour at 4°C. Beads were collected and washed 4 times with RIPA washing buffer (150 mM Tris–HCl, (pH 8.0), 150 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) NP-40). Immunoprecipitates were then resolved by 12.5% SDS-PAGE. The proteins resolved on the gel were transferred to a nitrocellulose membrane (0.45-μM pore size; Bio-Rad) by electroblotting. Then, the membrane was incubated with rabbit polyclonal antibodies against IN for 3 hours at room temperature and then it was probed at room temperature with horseradish peroxidase-linked donkey anti-rabbit antibodies (Amersham Biosciences) for 1 h. Finally, the membrane was washed extensively and protein-antibody interactions were visualized by the treatment with the sensitive enhanced chemiluminescence detection system (ECL detection kit, Amersham Biosciences), followed by exposure to KODAK Bio-Max Light Film (VWR).

2.7 Immunofluorescence assay.

COS-7 cells were grown on glass cover slips (12 mm^2) in 24-well plate for 24 hours and then transfected with different IN expression plasmids SVCMV-INwt/mut-YFP. After 48 hours, cells on the cover slip were fixed with PBS-4% paraformaldehyde for 5 min, permeabilized in PBS-0.2% Triton X-100 for 5 minutes and incubated with primary rabbit anti-GFP antibody followed by secondary FITC-conjugated anti-rabbit antibodies. Cells were viewed by using a confocal microscopy (Olympus IX-70) with a 50x oil immersion objective.

2.8 Single cycle replicating virus production and infection.

Production of different single-cycle replicating virus stocks and the measurement of virus titer were previously described (Ao et al., 2004). Briefly, 293T cells were co-transfected with NL4.3/luc/ΔBgl/ΔRI provirus, a VSV-G expressor and each of CMV-Vpr-RT-IN (wt/mut) expressor. After 48 hours, supernatants were collected and processed by ultracentrifugation through 40,000 x *g* for 2 hours to obtain virus stocks. Virus titers were quantified by RT activity assay(as indicated in **2.9**) (Yao et al., 1999).

To analyze the composition of virus, equal amounts of virus stock based on RT activity were lysed and directly loaded in 12.5% SDS-PAGE and virus composition was analyzed by western blotting with anti-HIV antibodies.

To test the effect of IN mutants on virus infection, dividing $CD4^+$ C8166 T cells were infected with equivalent amounts of VSV-G pseudotyped single cycle replicating viruses (5 cpm/cell) for 4 hours. Then, infected cells were washed and cultured in fresh RPMI media. At different times post-infection, $1x10^6$ cells from each sample were collected, washed twice with PBS, and lysed with 50 μl of luciferase lysis buffer (Fisher

Scientific Inc). Then, 10 or 20μl of cell lysate was subjected to the luciferase assay (as indicated in **2.10**). The infectivity of single cycle replicating virus was reflected by the luciferase activity.

2.9 RT activity assay

Fifty microliter of unconcentrated, but clarified HIV-1 culture fluid, mixed with 50μl of reaction buffer (50mM Tris Hydrochloride pH7.9, 5 mM $MgCl_2$, 150 mM KCl, 0.5 mM EGTA, 0.05% Triton-X-100, 2% Ethylene glycol, 5 mM DTT, 0.3 mM GSH, 50μg/ml poly-A-oligo(dT), 20μCi (^{3}H) dTTP) was incubated for 22 hours at 33°C. The reaction was stopped by adding 1ml cold 10% TCA (trichloroacetic acid) and precipitating 2 hours on ice. Then the reaction mixture was applied through G4 2.4 cm disks, which were rinsed briefly in 10% TCA, by vacuum filtration. The reaction tube was rinsed twice with cold 5% TCA, and all the washing solution was poured onto the disks as well. When no liquid remained, the sucking equipment was removed and the disks were recuperated in scintillation vials. The vials containing disks were dried at 60°C for 30 minutes followed by adding 5ml of scintillation liquid and then were ready for RT activity reading by scintillation spectrophotometry (Beckman coulter, LS6000TA).

2.10 Luciferase assay

Equal numbers ($1x10^6$) of cells infected with luc$^+$ HIV-1 viruses were collected at various time points, washed twice with washing buffer (sterile 1X PBS) and then lysed by 50 μl 1X CCLR (cell culture lysis regent) lysis buffer(25 mM Tris-phosphate pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N´,N´-tetraacetic acid, 10% glycerol and 1% Triton® X-100) (Fisher Scientific Inc). The cell lysate solutions were votexed for 10-15 seconds, and then centrifuged at 12000 x *g* for 15 seconds at room temperature or 2

minutes at 4°C. The supernatant, which contains cell lysate, was then transferred into a new microcentrifuge tube for luciferase activity testing. Briefly, the luciferase substrate–assay buffer mixture was prepared previously by adding the assay buffer to the lyophilized luciferase substrate (luciferin) and mixing well. At room temperature, 10-20 µl of cell lysate was added into polystyrene plate wells and mixed with 50µl luciferase substrate–assay buffer mixture. The luciferase activity was then measured and recorded by a Top-Count®NXT™ Microplate Scintillation & Luminescence Counter (Packard, Meriden) and the luciferase activity was read as relative light units (RLU).

2.11 Chromatin binding assay.

The association of HIV-1 IN with cellular chromatin was analyzed by a chromatin binding assay, as described previously (Llano et al., 2006b). Briefly, 293T cells were transfected with different SVCMV-HA-IN mutants. After 36-40 hours of transfection, cells were lysed for 15 minutes on ice in cold CSK I buffer (10 mM Pipes (pH 6.8), 100 mM NaCl, 1 mM EDTA, 300 mM sucrose, 1 mM $MgCl_2$, 1 mM DTT) supplemented with 0.5% (v/v) Triton X-100 and protease inhibitors. Cell lysates were centrifuged at 500 x *g*, 4°C for 3minutes to separate Triton-soluble (S1) and non-soluble (P1) fractions. Half of each S1 fraction was further lysed in RIPA buffer (150 mM Tris–HCl, (pH 8.0), 150 mM NaCl, 0.5% DOC, 0.1% (w/v) SDS, 1% (v/v) NP-40). The P1 fraction, which contains chromatin-bound, nuclear matrix-bound and insoluble proteins, was divided into two equal portions. One part was re-suspended in RIPA buffer (the P1 fraction). Another portion was re-suspended in CSK II buffer (10 mM Pipes (pH 6.8), 50 mM NaCl, 300 mM sucrose, 6 mM $MgCl_2$, 1 mM DTT) and treated with DNase (10 unit) for 30 minutes followed by extraction with 250 mM $(NH_4)_2SO_4$ for 10 minutes at 25° C, then

centrifuged at 1200 x *g* for 6 min at 4°C. The supernatant (S2 fraction, containing DNase-released chromatin-associated proteins) and pellet (P2, containing insoluble, cytoskeletal, and nuclear matrix proteins) were collected and added to RIPA buffer. All fractions were then analyzed by immunoprecipitation using anti-HA antibodies and detected by western blot with the same antibody.

CHAPTER 3 RESULTS

3.1 Expression of HIV-1 IN in *S. cerevisiae* HP16 strain induces a lethal phenotype.

Previous studies have shown that HIV-1 IN induces a lethal phenotype in some yeast strains, including JSC 302, W839-5C, and AB2 strains, but not in W303-1 strain (Caumont et al., 1996). In this study, we tested the lethal activity of HIV-1 IN in *S. cerevisiae* HP16 strain, that has a similar genotype to JSC 302 (Caumont et al., 1996). To do so, a yeast expression plasmid encoding a HIV-1 IN cDNA under the control of the galactose-inducible GAL1 promoter named p424Gal1-IN was constructed and transformed into *S. cerevisiae* HP16 yeast cells that were cultured in inducible (Trp^-, 2% galactose(Gal^+)) or non-inducible media (Trp^-, 2% raffinose(Raf^+)). The empty vector p424Gal1 plasmid was used as control. After 6 hours of galactose induction and non-induction, equal amounts of yeast cells were lysed and subjected to immunoprecipitation with anti-HIV antibody followed by western blotting using anti-IN antibodies, as described in Materials and Methods (**2.6**). Results clearly revealed that IN expression was only detected in p424Gal1-IN-transformed yeast cells (lane 2) under inducible conditions (Fig.3.1 A).

To test whether the expression of IN may affect yeast growth, the p424Gal1-IN- and p424Gal1-transformed HP16 yeast cells were grown in either non-inducible (Trp^-, 2% raffinose(raf^+)) or inducible media (Trp^-, 2% gal^+). After one day of cultivation, yeast growth was monitored. In the non-inducible media (Trp^-, 2% raf^+), both yeast cell cultures showed comparable growth capacity (Fig.3.1B, left panel). However, in the inducible media, yeast cells transformed with p424Gal1-IN exhibited a significant growth

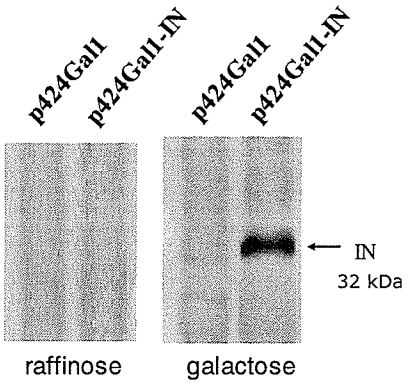


Figure3.1A Expression of HIV-1 IN in yeast strain HP16. Yeast cells transformed with IN expressing plasmid p424Gal1-IN or empty vector p424Gal1 were grown in inducible (Trp-, 2% gal+) or non-inducible selective media (Trp-, 2% raf+) for 6 hours. Cells were then lysed and subjected to immunoprecipitation with anti-HIV serum followed by WB with anti-IN antibody. Expressed IN band is shown and the molecular weight is indicated.

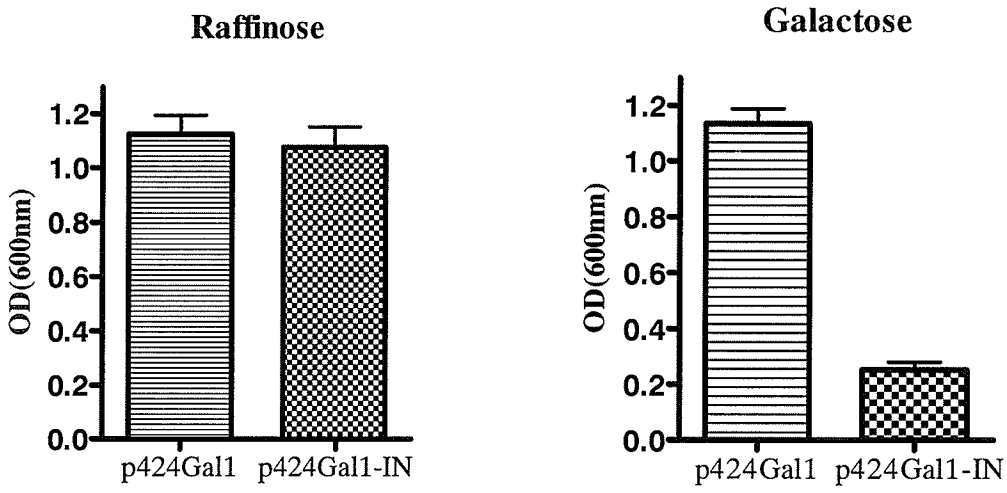


Figure3.1B The effect of HIV-1 IN on yeast growth by liquid assay. Equal starting amounts of p424Gal1-IN or p424Gal1 transformed yeast cells ($2x10^4$ cells) were grown in non-inducible selective media (Trp^-, 2% raf^+) (left panel) or in the inducible selective media (Trp^-, 2% gal^+) (right panel) at 30 °C for 24 hours. Yeast growth was monitored by measuring each yeast cell culture density by spectrophotometric analysis. Means and standard deviations from three independent experiments are shown.

inhibitory effect (Fig.3.1B, right panel). In parallel, the IN-induced yeast growth arrest was also tested in solid agar plates by "drop test", as described in the Materials and Methods (**2.5**). Similar to the results obtained in the liquid assay (Fig.3.1B), the p424Gal1-IN-transformed yeast cells lost growth ability when grown on IN-inducible selective agar plate (Trp^-, 2% gal^+) (Fig.3.1C, right panel) but grew well in the non-inducible condition (Fig.3.1C, left panel). These results clearly showed that yeast strain HP16 growth is also sensitive to HIV-1 IN.

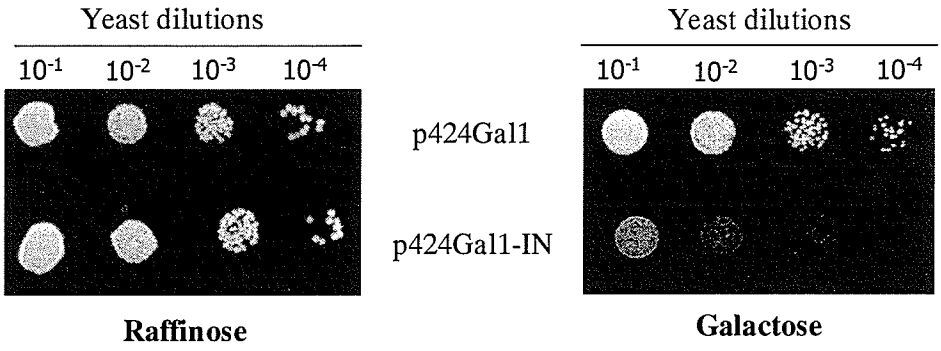


Figure3.1C The effect of IN expression on yeast growth by "drop test". Equal amounts of each transformed yeast cells were serially diluted and spotted onto either non-inducible agar plates (left panel) or inducible agar plate (right panel). After incubation for 3 to 5 days, yeast growth was recorded by photograph. The data are representative of three independent experiments.

3.2 HIV-1 IN-induced lethal phenotype in yeast strain HP16 is independent of its catalytic activity.

We next tested whether IN-induced lethality in yeast strain HP16 was dependent of its catalytic activity or not. To do so, different IN active-site missense mutants including the well documented single mutant D64E, D116A, and double mutant D64E/D116A were introduced into the p424Gal1-IN yeast expression plasmid. Then we used these class I IN catalytic mutants in experiments similar to those described above. We found that, as shown in Fig. 3.2A, similar levels of each IN mutant D64E, D116A, and double mutant

D64E/D116A were detected in HP16 yeast as compared to the wild type IN. That means catalytic IN mutants did not affect the protein expression.

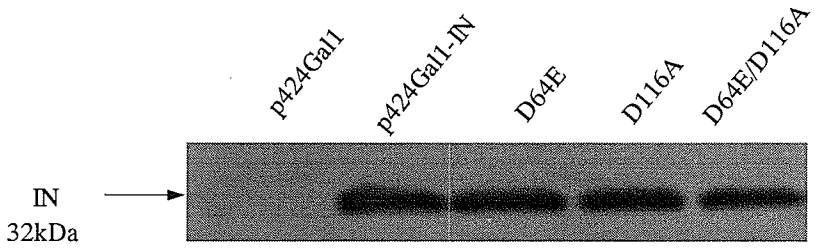


Figure3.2A Comparison of expression levels of HIV-1 catalytic IN mutants with wild type IN in yeast strain HP16. Yeast cells transformed with different catalytic IN mutants, wild type IN or empty vector p424Gal1 were grown in an inducible selective media (Trp^-, 2% gal^+) for 6 hours. Cells were then lysed and subjected to immunoprecipitation with anti-HIV serum followed by WB with anti-IN antibody. Molecular weight and IN bands were shown.

To test whether these IN catalytic mutants could abolish IN lethal activity in yeast, we cultured the same amounts of HP16 yeast cells ($2x10^4$) transformed with each catalytic mutant in IN-inducible (Trp^-, 2% gal^+) and/or IN non-inducible media (Trp^-, 2% raf^+). Also, yeast cells transformed with the wild type (wt) IN and empty p424Gal1 vector were used as positive control and negative control respectively. Result in Fig.3.2B displayed that in non-inducible media (Trp^-, 2% raf^+), yeast cells transformed with either wt IN or each IN mutant showed comparable growth capacity (Fig.3.2B, upper panel). However, when grown in the inducible media (Trp^-, 2% gal^+), all yeast cells transformed with wt IN or each catalytic IN mutant exhibited a significant growth defect when compared to cells transformed with the p424Gal1 control plasmid (Fig.3.2B, lower panel).

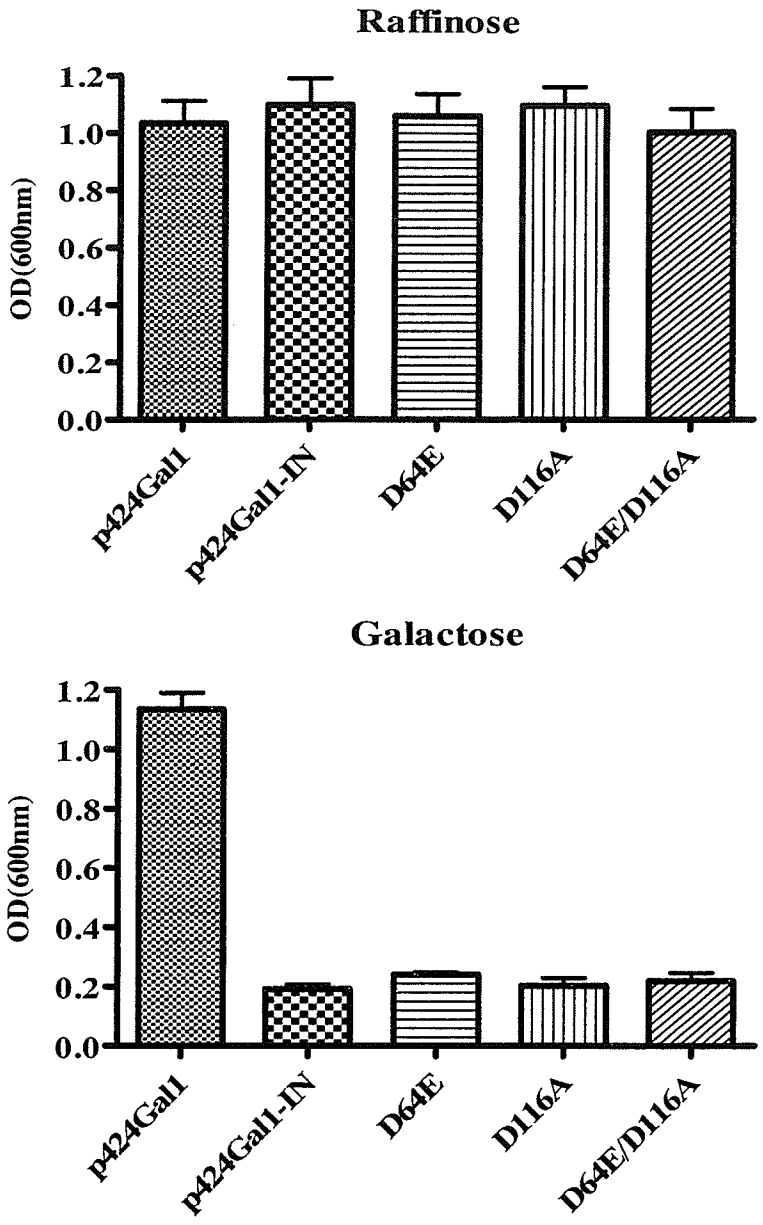


Figure3.2B Effects of catalytic IN mutants on yeast growth by liquid assay. Three indicated IN catalytic mutants were transformed into yeast strain HP16. Then the growth effect was measured as indicated as Figure3.1B. Means and standard deviations from three independent experiments are shown.

Meanwhile, similar amounts of serially diluted yeast cells transformed with wt IN and each catalytic IN mutant were spotted onto IN inducible (Trp^-, 2% gal^+) and non-inducible (Trp^-, 2% raf^+) agar plates. Results showed that none of these three catalytic mutants could abolish or alleviate IN-induced lethal phenotype as compared to the wild

type IN (Fig.3.2 C). All together, these results clearly indicated that HIV-1 IN-induced lethal phenotype was not affected by three catalytic IN mutants, suggesting this IN lethal activity in yeast HP16 is not dependent on its catalytic activity.

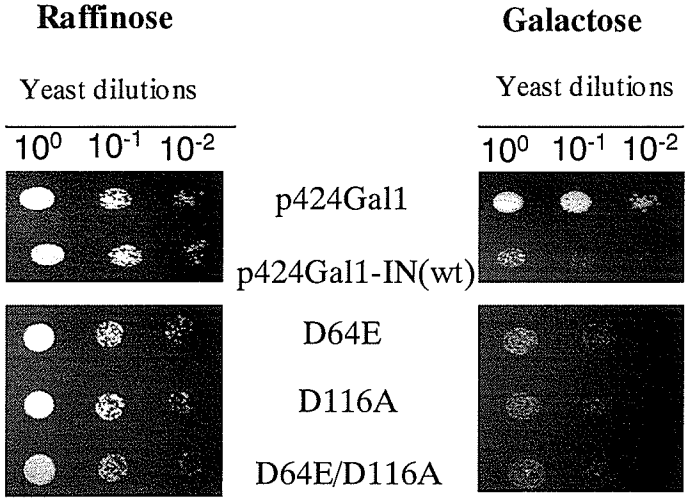


Figure3.2C The effect of catalytic IN mutants on yeast growth by "drop test". Experimental procedures are identical as indicated in Figure3.1C. These data are representative of three independent experiments.

3.3 Reduced expression level of catalytic IN mutant in yeast strain HP16 does not abolish the lethal phenotype.

High level expression of HIV-1 IN in yeast or mammalian cells could be toxic to the cells and then subsequently affect the cell growth. This kind of cell growth defect may not be directly correlated with IN's catalytic activity. Therefore, to rule out the possibility that the lethal phenotype observed with catalytic mutants may be due to the high level expression of the protein in yeast, we cultured yeast cells transformed with wt IN and catalytic IN mutant D64E/D116A in IN-inducible media containing different concentrations of galactose (decreased from 2% to 0% galactose), as indicated in Fig.3.3A. Our results clearly showed that the IN expression was reduced as a result of decreased galactose concentration (Fig. 3.3A). However, it still did not reveal the

attenuated lethal activity of IN mutant (D64E/D116A) compared with wild type IN (Fig.3.3B). Even under the condition of the lowest protein expression (0.05% galactose concentration), catalytic IN mutant D64E/D116A still can efficiently induce the lethal effect. Taken together, all of these results indicated that IN-induced lethal phenotype in yeast strain HP16 is not mediated by its catalytic activity.

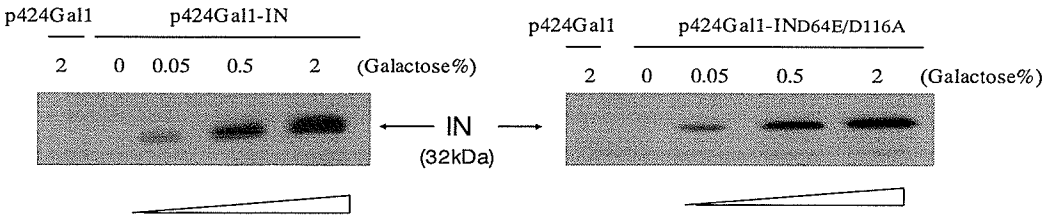


Figure3.3A Reduced expression level of wt/mut IN under decreased galactose concentration in yeast strain HP16. Equal amounts of yeast HP16 cells transformed with wt IN and catalytic IN mutant D64E/D116A were grown in IN-inducible media containing different concentration of galactose (from 0% to 2% galactose). After 6 hours, each cell population was then lysed and subjected to immunoprecipitation with anti-HIV serum followed by WB with anti-IN antibody.

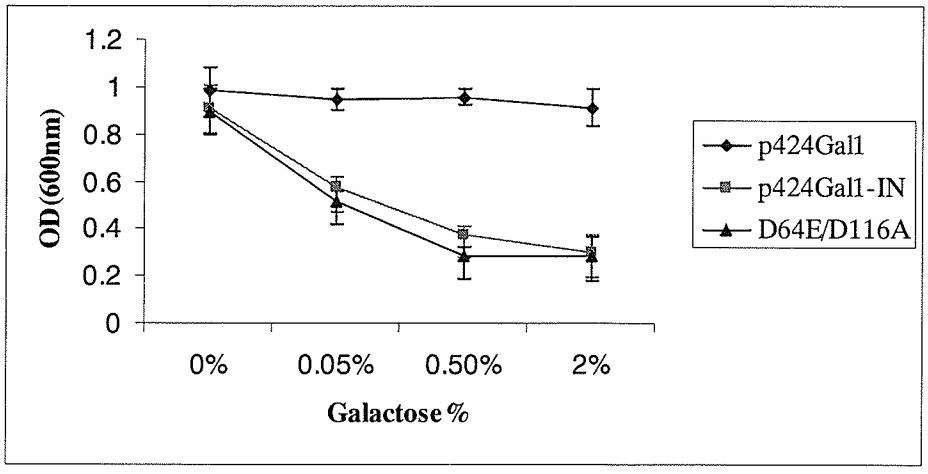


Figure3.3B The effect of reduced IN expression on yeast growth. Equal amounts of yeast HP16 cells ($2x10^4$) transformed with wt IN and catalytic IN mutant D64E/D116A were grown in IN-inducible media containing different concentration of galactose (from 0% to 2% galactose) at 30 °C for 24 hours. Yeast growth was monitored by measuring each yeast cell culture density by spectrophotometric analysis. Means and standard deviations from three independent experiments are shown.

3.4 Knockout of RAD52 does not increase the sensitivity of yeast to HIV-1 IN's effect.

Even though the results above did not reveal the importance of the catalytic site of HIV-1 IN for its induced lethal effect in yeast, we still could not rule out this effect was due to IN's DNA damage activity, as proposed before (Caumont et al.1996). To test this, we have checked whether knockout of *rad52* gene would sensitize yeast to IN lethal activity. The *rad52* gene encodes one component of DNA repair system molecules, RAD52, which is specifically involved in the repair of double-strand DNA breaks (Mortimer et al. 1981; Barnes and Rine 1985) and may be necessary for the normal mitotic recombination event. In this experiment, we selected two yeast strains BY4743 (*rad52*$^{+}$) and BY4743 (*rad52*$^{-}$). Both of them are diploid yeast strains and the only difference between them is two *rad52* genes have been knocked out in the yeast strain BY4743 (*rad52*$^{-}$).

For these two yeast strains, we constructed another IN yeast expression plasmid (p423Gal1-IN) and also introduced some IN mutants into this vector including D116A and D64E/D116A. Then, we performed the same liquid assay and "drop test" as described above to investigate the effect of RAD52 on yeast growth in these two strains. Results shown in Fig3.4A,B clearly revealed that HIV-1 IN can induce the lethal phenotype no matter whether *rad52* gene is deleted or not. Moreover, the two IN catalytic mutants D116A, D64E/D116A showed the lethal activity as efficiently as wild type IN and this also confirmed the results we described in the HP16 strain. In contrast, comparing the growth activity between yeast strain BY4743 (*rad52*$^{+}$) and BY4743 (*rad52*$^{-}$) transformed with wt and catalytic IN mutants, there was no significant difference.

Taken together, in these two yeast strains, RAD52 does not increase the sensitivity of yeast to HIV-1 IN's lethal effect.

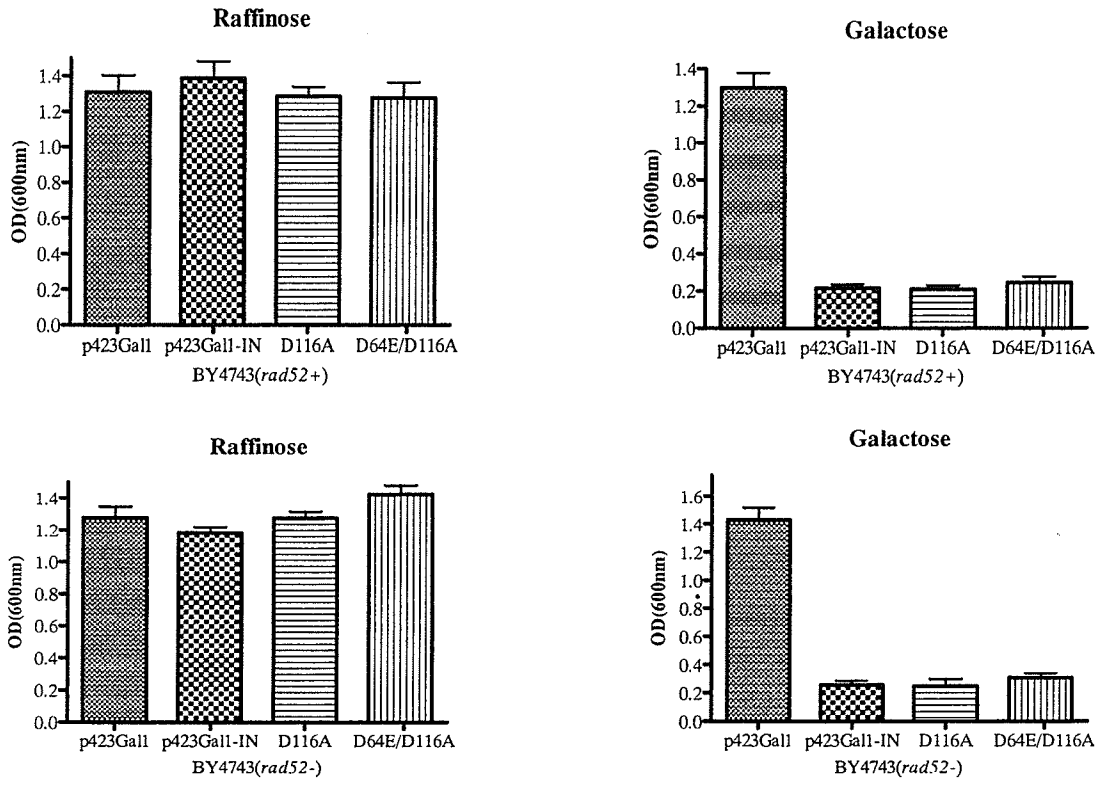
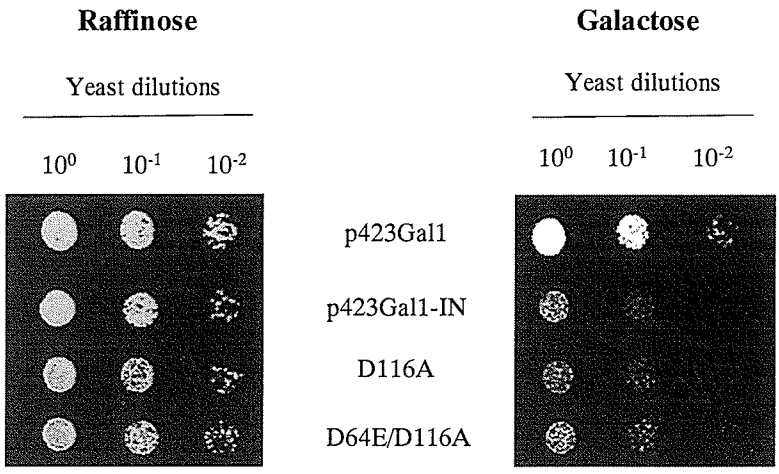


Figure3.4A Effects of RAD52 on HIV-1 IN-induced lethal phenotype by liquid assay. BY4743 (*rad52*$^+$) and BY4743 (*rad52*$^-$) yeast cells were transformed with wild type IN and two indicated catalytic IN mutant expressors (p423Gal1-IN$_{wt/mut}$). Each transformed yeast population was first grown in non-inducible selective media (His$^-$, 2% raf$^+$) overnight. Then equal amounts of transformed yeast cells were grown in non-inducible (His$^-$, 2% raf$^+$)or inducible selective media (His$^-$, 2% gal$^+$) at 30 °C for 24 hours. Yeast growth was monitored by measuring each yeast cell culture density. Means and standard deviations from three independent experiments are shown.

Yeast strain BY4743 (*rad52*$^+$)



Yeast strain BY4743 (*rad52*$^-$)

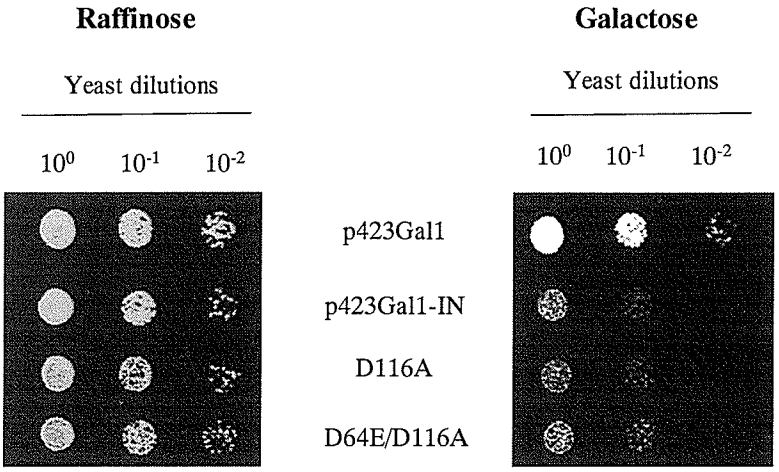


Figure3.4B Effects of RAD52 on HIV-1 IN-induced lethal phenotype by "drop test". The effect of RAD52 on IN-induced lethal phenotype was also tested by "drop tests" as described as Figure3.1C except that using different selective agar plates (His$^-$, 2% raf$^+$ or 2% gal$^+$).

3.5 Characterization of the lethal activity of different HIV-1 IN mutants in yeast strain HP16.

We next tried to identify the critical amino acid(s) or motif of IN that is involved in the lethal phenotype. Variant IN mutants, including F1A, K136A, K159P, V165A, A179P, KR186,7AA, KK215.9AA and RK263,4AA, as indicated in Fig.3.5A, were introduced in each functional domain of IN and tested for their effects on IN activity in yeast.

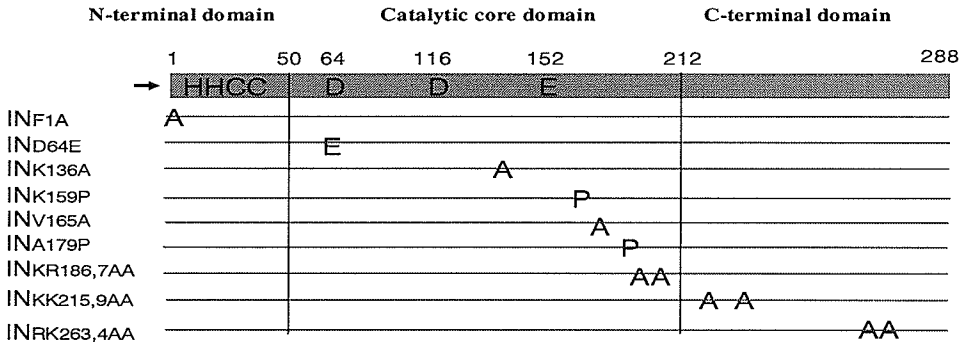


Figure3.5A Introduction of specific point mutations in different IN domains As indicated, 9 different substitution mutation clones distributed on the different IN domains were generated by a two-step mutagenic PCR method and then were introduced into the yeast expressing plasmid p424Gal1-IN. All IN mutants were subsequently analyzed by DNA sequencing to confirm the presence of mutations.

Some mutants, such as V165A, K186Q, KK215.9AA and RK263,4AA, were previously shown to disrupt viral replication at different step including proviral DNA integration (Ao et al., 2005; Limon et al., 2002b; Lu et al., 2005; Lu et al., 2004; Petit et al., 2000). In parallel, an IN class I mutant D64E was also included in this study, since this mutant is unable to mediate HIV integration by specifically blocking the catalytic activity of IN. The expression of wild type IN and IN mutants (D64E, K136A, K159P, V165A, A179P, KR186,7AA, KK215.9AA and RK263,4AA) in yeast was evaluated and

revealed that under inducible conditions, similar protein expression levels were detected for the wild type IN and each mutant in the corresponding yeast population (Fig.3.5B, upper panel; lanes 2-10). To ensure the similar amounts of cell lysates were used for IN's detection, the endogenous yeast β-Actin was also detected by using western blotting with anti-Actin antibodies, and results showed that similar amounts of endogenous β-Actin were present in the lysate samples (Fig.3.5B, lower panel).

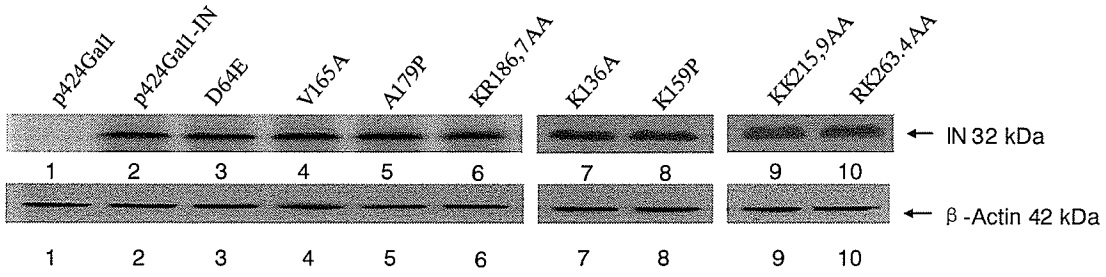


Figure3.5B Expression of different IN mutants in HP16 yeast cells. Expression of different IN mutants in yeast strain HP16 was tested as indicated in Figure3.1A (upper panel). Also, equal amounts of cell lysates were loaded directly on SDS-PAGE and endogenous yeast β-Actin was detected by anti-Actin western blotting (lower panel).

To test the effect of each IN mutant on yeast growth, equal amounts of yeast cells were cultured in inducible or non-inducible media and the growth of each yeast population was monitored by liquid assay and the "drop test". In non-inducible media, all IN mutants grew to the same extent (Fig3.5 C, upper panel; Fig.3.5 D, left panel). However, in the IN-inducible media, yeast cells transformed with different IN mutants showed various growth capabilities (Fig.3.5C, lower panel; Fig.3.5D, right panel). In particular, cells transformed with IN mutants V165A, A179P or KR186,7AA grew to similar level as yeast cells transformed with empty vector (Fig.3.5C, lower panel; Fig.3.5D, right panel). Therefore, we designated these three mutants as yeast lethal

phenotype-defective IN mutants. Overall, we demonstrated that three IN mutants including V165A, A179P or KR186,7AA, which are located in the C-terminal region of IN core domain, almost lost the ability to induce a lethal phenotype in HP16 yeast strain.

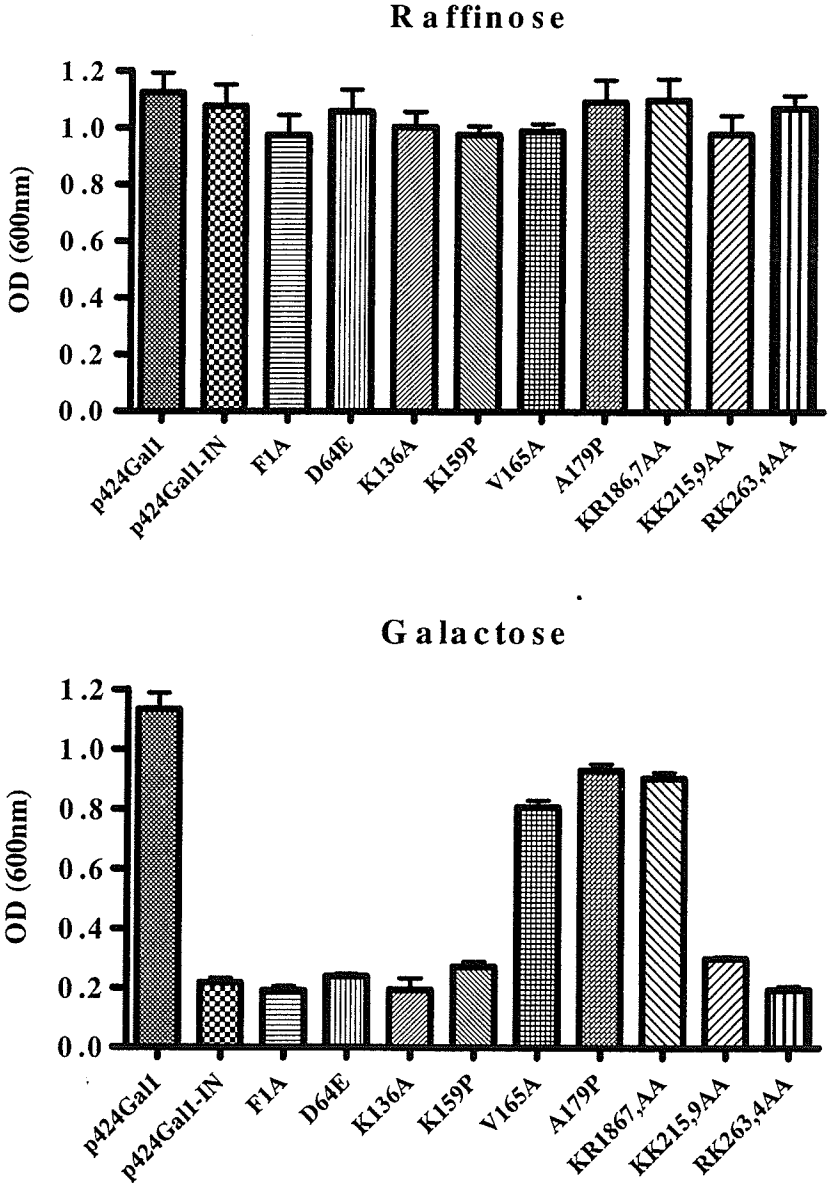


Figure3.5C Effects of different IN mutants on yeast growth by liquid assay. Experimental procedures are indicated as Figure3.1B. Means and standard deviations from three independent experiments are shown.

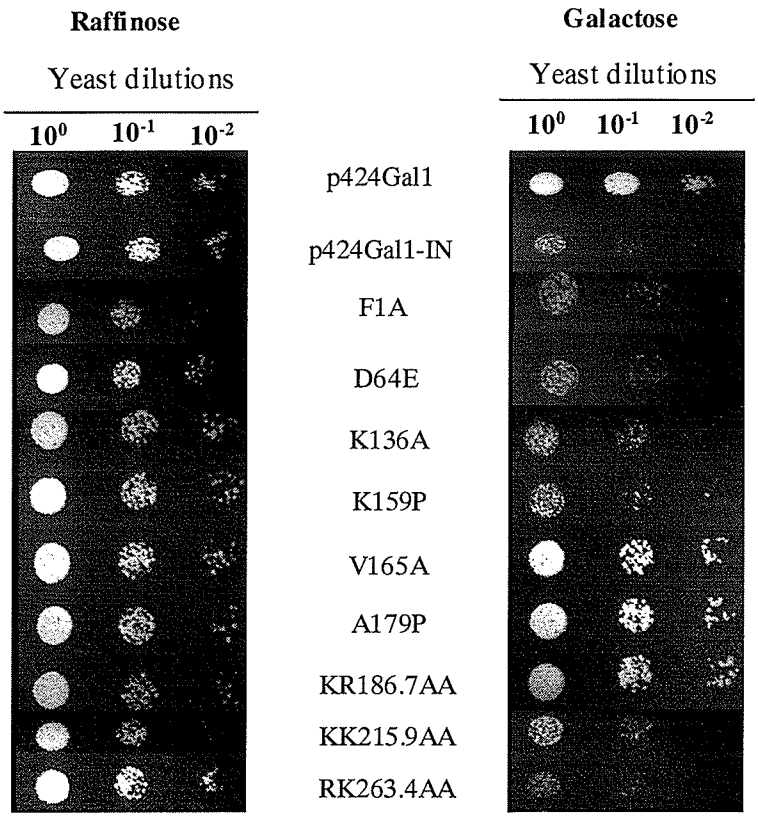


Figure3.5D Effects of different IN mutants on yeast growth by "drop test". Similar experimental procedures are described as in Figure3.1C. The data are representative of three independent experiments.

3.6 The effect of yeast lethal phenotype -defective IN mutants on HIV-1 single-cycle replication.

Given that these mutants drastically affected IN's activity in yeast, we next evaluated their effects on HIV-1 replication. The non-lethal IN mutants (V165A, A179P and KR186,7AA) were introduced into a previously described HIV-1 single-cycle replication system (Ao et al., 2005). Briefly, each IN mutant was first inserted into a Vpr-RT-IN expressor and named Vpr-RT-INV165A, Vpr-RT-INA179P and Vpr-RT-INKR186,7AA. Then, each Vpr-RT-IN mutant expressor was co-transfected in 293T

cells with a RT/IN/Env gene-deleted HIV-1 provirus NL4.3/luc/ΔBgl/ΔRI, and a vesicular stomatitis virus G (VSV-G) glycoprotein expressor to produce single cycle replicating viruses. This process can be simplified as indicated in Fig.3.6 A. After 48 hours of transfection, VSV-G pseudotyped single cycle replicating viruses were harvested and concentrated by ultracentrifugation.

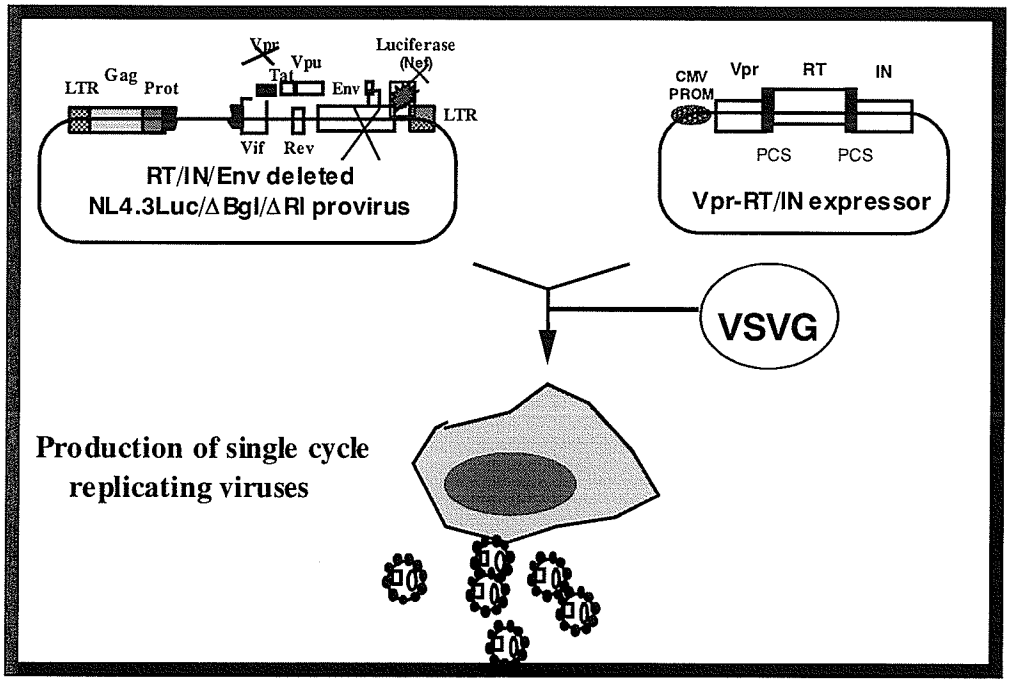


Figure3.6A Diagram of single cycle replicating viruses production.

To evaluate the trans-incorporation of RT and IN in VSV-G pseudotyped viral particles, similar amounts of each virus stock were lysed and directly loaded in SDS-PAGE for virus composition analysis by western blotting with anti-HIV antibodies. The result showed that each IN mutant virus contained similar levels of IN, RT, and Gag-p24, as compared to the wild-type viruses (Fig.3.6B, lane 1 to 6), indicating that trans-

incorporation of RT and IN as well as HIV-1 Gag processing was not differentially affected by different IN mutants.

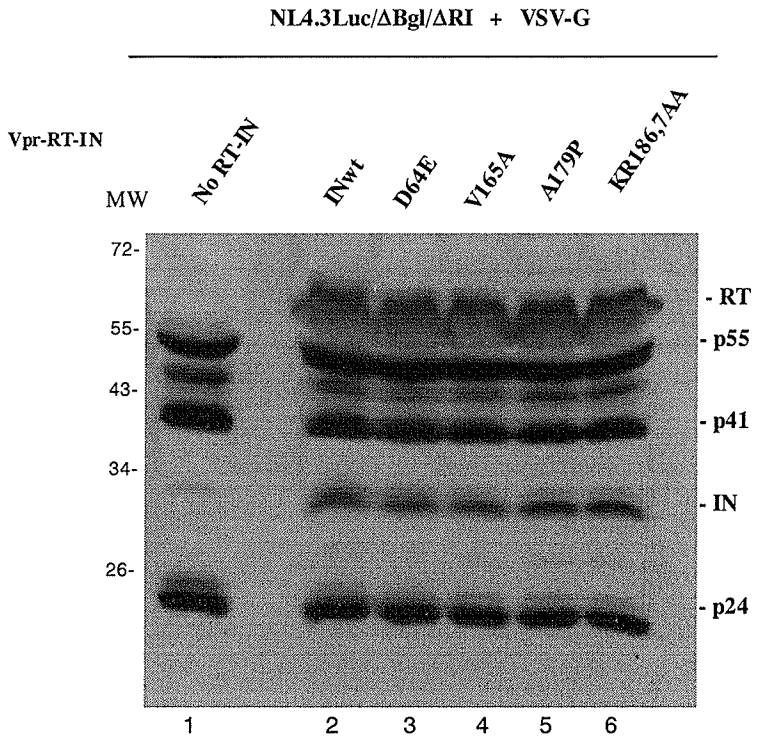


Figure3.6B Composition analysis of single cycle replicating IN mutant viruses. The equal amounts of produced virus stocks (lane 1 to 6) were lysed and directly loaded in 12.5% SDS-PAGE and analyzed by Western blot with human anti-HIV serum. The positions of HIV-1 Gag, RT and IN proteins were indicated at the right side of gel.

To check the infectivity of different IN mutant viruses in $CD4^+$ T cells, we infected C8166 $CD4^+$ T cells with equal amounts of VSV-G pseudotyped IN mutant virus (at 5 cpm of RT activity/cell). Since all IN mutant viruses contained a luciferase (luc) gene in place of the *nef* gene, viral infectivity was monitored by using a sensitive luc assay (Ao et al., 2005). Results showed that the wild type IN virus infection induced a high level of luc activity and the peak level (1.5 $x10^5$ RLU) was detected at 64 hours post-infection in dividing C8166 T cells (Fig.3.6 C). The class I mutant D64E virus had only a basal level of luc activity which was approximately 10^4-fold lower than the wild type level (Fig.3.6

C). Interestingly, when C8166 cells were infected with VSV-G pseudotyped viruses containing yeast lethal phenotype-defective IN mutants, the levels of luc activity detected were similar to that obtained with the D64E mutant throughout a 6 day period (Fig.3.6 C). The results clearly indicated that, like the class I D64E mutant virus, all three mutants V165A, A179P and KR186,7AA are drastically attenuated for infectivity and are unable to replicate in C8166 T cells.

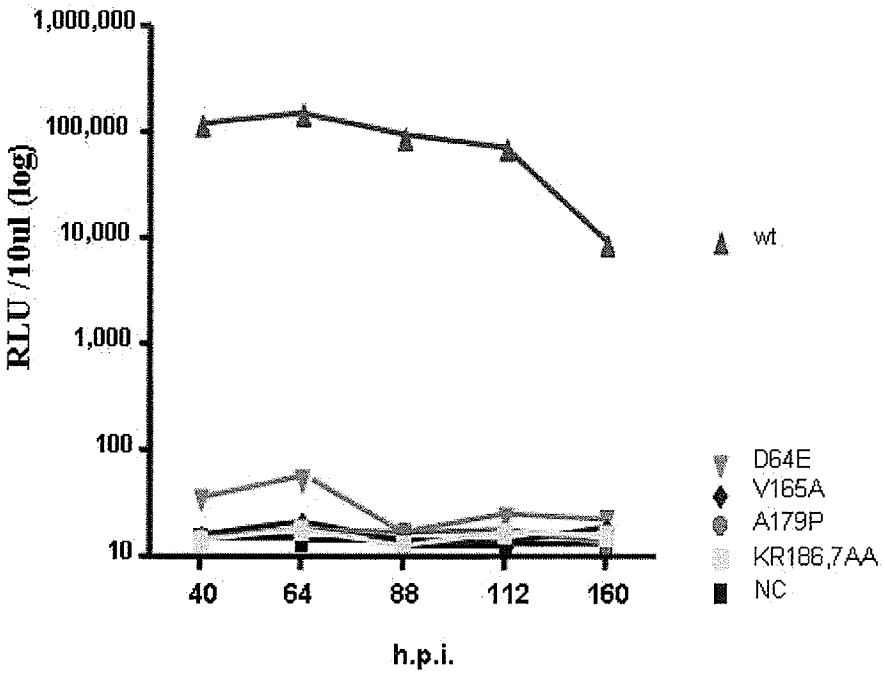
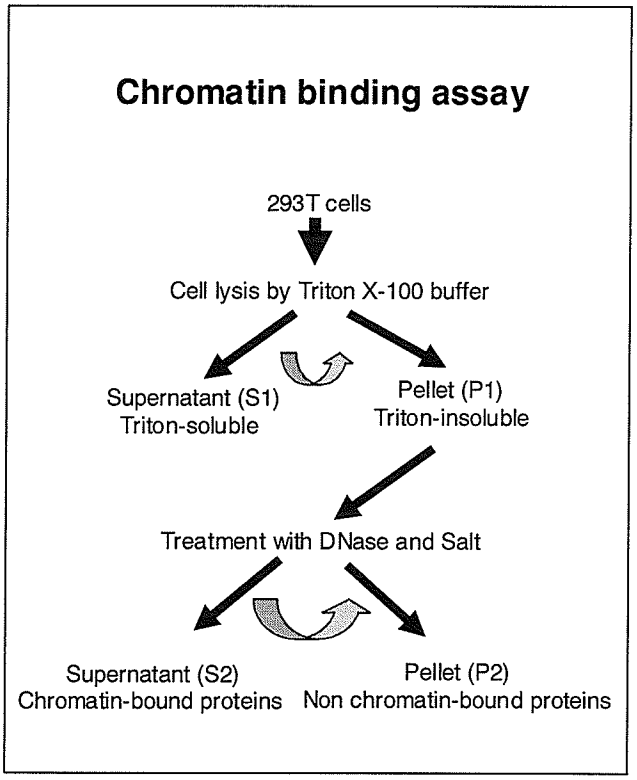


Figure3.6C Effect of yeast lethal phenotype-defective IN mutants on VSV-G pseudotyped HIV-1 single cycle replication. To assess viral infection, equal amounts of VSV-G pseudotyped viruses were used to infect the dividing CD4+ C8166 T cells. At different time intervals, the same amounts of cells were collected and the infection mediated by each virus stock was evaluated by luciferase assay. The data is representative of results obtained in three independent experiments. NC: negative control; h.p.i.: hours of post infection.

3.7 The yeast lethal phenotype -defective IN mutants translocated to the nucleus but lost their ability to bind chromatin.

The step(s) at which these IN mutants affect replication during HIV-1 infection are not defined. Given that the IN class I mutant D64E still induced lethal phenotype in HP16 yeast strain, it is possible that these lethal phenotype-defective IN mutants disrupt a particular activity of IN other than the catalytic activity. Therefore, we further analyzed the association of different IN mutants with host chromatin. The 293T cells were transfected with SVCMV-HA-IN expressor in which cDNAs encoding for each of the wild type IN or different mutants (D64E, K136A, V165A, A179P and KR186,7AA) were inserted inframe at the 3' end of a hemagglutinin (HA)-tag. The presence of HA-IN in the chromatin-bound and non-chromatin-bound fractions was subsequently analyzed by a chromatin binding assay, as described in Materials and Methods (**2.11**). Fraction processing is shown in Fig.3.7 A. As a control, the YFP protein was expressed in the 293T cells and cells were processed in an identical manner and analyzed for the association of YFP with the host chromatin.



Figurre3.7A Fractionation patterns of HIV-1 IN in a chromatin-binding assay. Cell fraction processing was summarized and detailed procedures as indicated in Materials and Methods (**2.11**). Modified from Manuel Llano, 2006

Results revealed that for the wild type HA-IN, mutant HA-IND64E and HA-INK136A, up to 20-25% of HA-IN was found in the chromatin-bound (P1) fraction (Fig.3.7B, upper panel). However, mutants HA-INV165A, HA-INA179P and HA-INKR186,7AA were solely present in the non chromatin-bound S1 fraction (Fig.3.7B, upper panel, comparing S1 to P1, lanes 5 to 7). As expected, the YFP was also only detected in the non chromatin-bound (S1) fraction (Fig.3.7B, lower panel). To ensure that the wild type HA-IN, HA-IND64E and HA-INK136A were indeed associated with cellular chromatin, the P1 fractions were treated with DNAse/salt. As expected, results showed that the DNAse treatment released HA-IN, HA-IND64E and HA-INK136A from

the P1 fraction into the soluble fractions (S2). These results suggest that yeast lethal phenotype-defective IN mutants are unable to associate with the host cell chromatin.

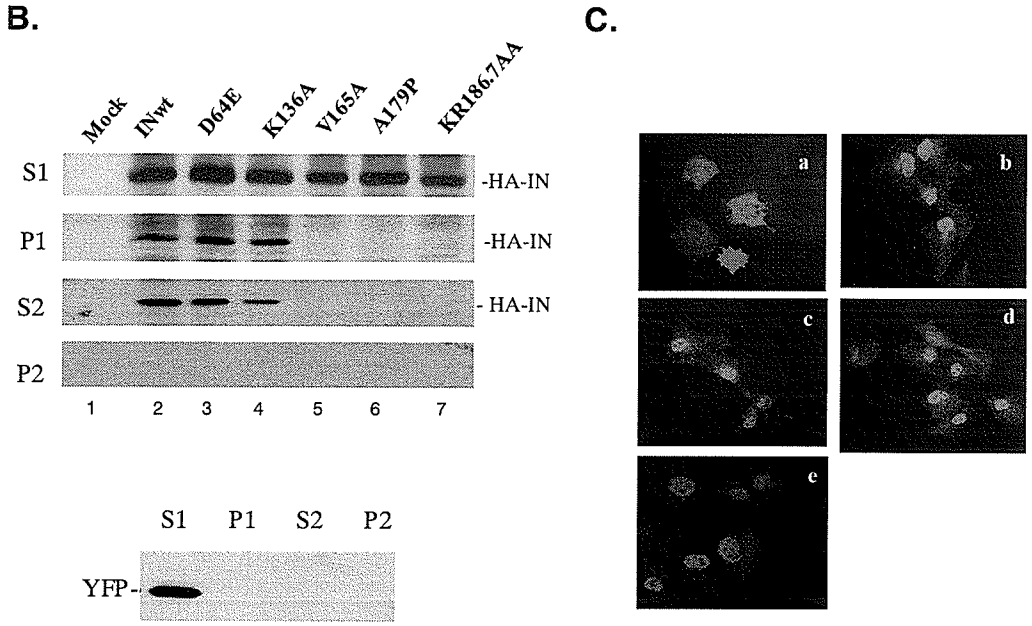


Figure3.7 B,C The yeast lethal phenotype-defective IN mutants lost their chromatin binding ability while retained their nuclear localization. B). 293T cells were transfected with different SVCMV-hemagglutinin (HA) tagged-IN expressors (including the wild type IN and mutants, as indicated). After 48 hr, transfected cells were lysed in cold CSK I buffer (0.5% Triton X-100), fractionated and the presence of HA-IN in the chromatin-bound and non-chromatin-bound fractions was analyzed by immunoprecipitation and western blot with anti-HA antibodies. In parallel, the presence of GFP in different fractions of 293T cells transfected with a SVCMV-YFP expressor was also analyzed using identical procedure and anti-GFP antibodies (lower panel). S1: Supernatant (non-chromatin-bound fraction); P1: Pellet (chromatin-bound fraction); S2: Chromatin-bound proteins in P1; P2: Non-chromatin–bound proteins in P1. C). Intracellular localization of different IN mutants. COS-7 cells were transfected with different IN-YFP fusion protein expressors and subjected to anti-GFP indirect immunofluorescence analysis. a. YFP; b. INwt-YFP; c. INV165A-YFP; d. INA179P-YFP; e. INKR186,7AA-YFP.

To rule out the possibility that the inability of the IN mutants to bind to host chromatin was due to an exclusion of the protein from the nucleus, we determined the

intracellular localization of each IN mutant in COS-7 cells, given that COS-7 cells have well-defined morphology and are suitable for observation of intracellular protein distribution (Yao et al., 1995). We constructed different IN-YFP fusion proteins by fusing each IN mutant with YFP to avoid the passive diffusion of small size protein into the nucleus (Ao et al., 2005). Each IN-YFP expressor was transfected into COS-7 cells. After 48 hr, cells were fixed and subjected to indirect immunofluorescence using anti-YFP antibody. Results showed that, in contrast to a diffused intracellular localization pattern of YFP (Fig.3.7C, a), all of IN-YFP fusion proteins, including the wild type IN and lethal phenotype-defective mutants, were predominantly localized in the nucleus (Fig.3.7C, b to e). These results indicated that, while, the three yeast lethal phenotype-defective mutants have the ability to translocate into the nucleus like the wild type IN, they are defective in their ability to bind to cellular chromatin.

3.8 Replication defect of yeast lethal phenotype-defective IN mutant viruses.can be partially complemented by D64E mutant.

Data accumulated so far indicated that the yeast lethal phenotype-defective IN mutants and class I D64E appear to affect different steps of viral replication. So, it should be possible that the replication defect of these lethal phenotype-defective IN mutant viruses might be complemented by D64E mutant. To test this possibility, we co-transfected 293T cells with HIV-1 provirus NL4.3lucΔBglΔRI, VSV-G expressor, and a mix of different Vpr-RT-IN mutants, as indicated in Fig.3.8 (right panel). After 48 hours of transfection, VSV-G pseudotyped viruses were collected and equal amounts of viruses (5 cpm of RT activity per cell) were used to infect CD4+ C8166 T cells.

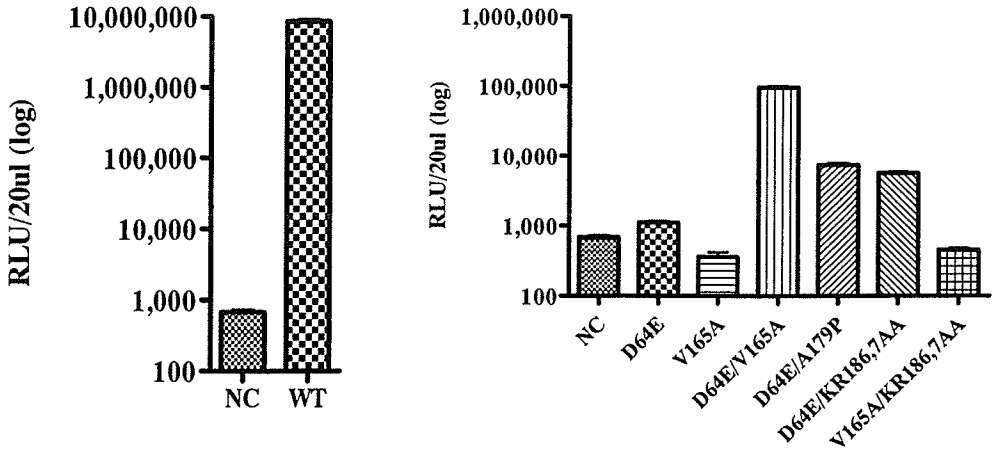


Figure3.8 Complementation of chromatin binding-defective IN mutant viruses with D64E mutant. To test the functional complementation of IN mutants, different combination Vpr-RT-IN mutant expressors, as indicated, were co-transfected with RT/IN/Env gene-deleted HIV-1 provirus and VSV-G expressor in 293T cells. At 48 hr post-transfection, VSV-G pseudotyped viruses were collected from the supernatant by ultracentrifugation followed by quantification with measurement of virion-associated RT activity. To test the infection of each virus stock, equal amounts of viruses were used to infect dividing CD4+ C8166 T cells, and at 72 hr of infection, cells were collected and the cell-associated luciferase activity was measured. NC: negative control. Error bars represent variation between duplicate samples and the results are representative of three independent experiments.

Results revealed that, while wild type virus infection resulted in a high level of luc activity ($8.5x10^6$ RLU/20 µl), the D64E and V165A mutant viruses only induced a basal level of luc activity (Fig.3.8, right panel). When C8166 cells were infected with viruses containing two IN mutant proteins, such as D64E/V165A, D64E/A179P, or D64E/KR186,7AA, there was a partial *trans*-complementation for virus infection as the luc activity was significantly higher, as compared to D64E and V165A virus infections (Fig.3.8, right panel). The result was further supported by the fact that there was no *trans*-complementation for V165A and KR186,7AA mutants (Fig.3.8, right panel). It should be noted that the D64E/A179P or D64E/KR186,7AA virus infection induced significantly lower luc activity as compared to D64E/V165A virus infection (Fig.3.8).

This may be due to a more profound effect of A179P or KR186,7AA mutants on other IN's functions. Nevertheless, these *trans*-complemention analyses provide evidence that the IN mutants V165A, A179P and KR186,7AA, which are located in the C-terminal region of IN core domain, affect chromatin-targeting of viral DNA that is critical for HIV integration and replication.

CHAPTER 4 DISCUSSION AND FUTURE DIRECTIONS

HIV-1 IN has multiple functions during the early phase of HIV-1 replication, including reverse transcription, viral DNA nuclear import and integration (Ao et al., 2005; Bukovsky and Gottlinger, 1996; Engelman et al., 1995; Gallay et al., 1997; Ikeda et al., 2004; Nakamura et al., 1997; Wu et al., 1999). Therefore, it is difficult to specifically assess one particular function of IN during viral replication due to the fact that most mutants of IN exhibit pleiotropic phenotypes. Various *in vitro* assays and *in vivo* analyses, including yeast IN expression systems like the one used here have been developed to assess different activities of HIV-1 IN. Previous studies have shown that the expression of functional IN in some yeast strains led to the emergence of a lethal phenotype that may be related to the HIV-1 integration reaction (Caumont et al., 1996). In comparison to prokaryotic systems, the yeast model is a useful tool to investigate IN activity during the integration process and the study of IN's activity in yeast may provide great insights into the mechanisms how HIV-1 IN interacts with cellular machinery. However, the molecular mechanism underlying this lethal activity of IN in yeast is still not fully understood. The previous report by Caumont *et al.* showed that the IN class I mutant D116A lost the lethal phenotype, suggesting a requirement for IN's catalytic activity (Caumont et al., 1996). Interestingly, a subsequent study revealed that the specific mutation targeting amino acid E152, another crucial residue of IN's catalytic triad motif (D64D116(35)E152) did not affect its lethal activity in yeast (Calmels et al., 2004). Thus, the IN-mediated yeast lethal phenotype appears to be a more complex process and the elucidation of this biological activity of IN in yeast may provide valuable insights into the understanding of IN's action during HIV-1 replication.

To further investigate the molecular basis underlying HIV-1 IN's lethal activity in yeast, we expressed different IN mutants in *S. cerevisiae* HP16 strain. Three HIV-1 IN mutants V165A, A179P and KR186,7AA did not induce the lethal phenotype in HP16 cells compared with wild type IN(Fig.3.5 C and D). Interestingly, our results also showed that some IN class I mutants including D64E, D116A, and D64E/D116A were fully capable of inducing the lethal phenotype when they were introduced in HP16 cells (Fig.3.2 B and C). This observation is consistent with the previous study by Calmels *et al* showing that the specific mutation targeting amino acid E152 still efficiently induced the lethal phenotype (Calmels et al., 2004). Moreover, in our studies, the reduced expression level of HIV-1 catalytic IN mutant and the RAD52 knock out did not increase the sensitivity of yeast to IN's effect (Fig.3.3 B and Fig.3.4 A, B). All these data suggest that the catalytic activity of IN is essential for its lethal effect in yeast strain HP16 and it can be speculated that some un-characterized activities of HIV-1 may play an important role for its lethal activity.

HIV-1 IN has been shown to be an important viral factor involved in HIV DNA binding to cellular chromatin and this IN-mediated chromosomal tethering function has been shown to be important for HIV-1 integration and for successful viral replication (Emiliani et al., 2005; Llano et al., 2006a; Maertens et al., 2003). In this study, the chromatin binding ability of different IN mutants was tested and our analysis demonstrate that, in contrast to the wild type IN and D64E mutant, V165A, A179P and KR186,7AA mutants lost their abilities to bind to host chromatin(Fig.3.7). To rule out the possibility that their inability to bind to cellular chromatin may be due to a defect at nuclear translocation step, we further showed that all three IN mutants can efficiently enter into

the nucleus (Fig.3.7). Thus, the chromatin-binding ability of HIV-1 IN is in fact involved in yeast lethality. To correlate the loss of IN's lethal ability in yeast with their effects on HIV-1 replication, we further tested the effect of yeast lethal phenotype-defective mutants V165A, A179P and KR186,7AA on HIV-1 single-cycle replication. The experiments clearly showed that, similar to the IN class I D64E mutant, all three yeast lethal phenotype-defective IN mutant viruses were replication deficient (Fig.3.6C). More interestingly, the evidence presented in this study clearly indicated that the infection defect of all three IN mutant viruses was partially restored to different extent by the presence of D64E mutant (Fig.3.8), suggesting that the action of V165A, A179P and KR186,7AA is different from the catalytic mutant. Indeed, our biochemical data further demonstrate that these three yeast lethal phenotype defective IN mutants, which are clustered in the C-terminal region of IN core domain, were unable to bind to the cellular chromatin while D64E does. Interestingly, it should be noticed that the complementation is efficient with IN mutant V165A but not with A179P and KR186,7AA. This could be explained by the profound effects of A179P and KR186,7AA during viral integration. Previous mutagenic study has shown that the amino acid lysine (K) 186 of HIV-1 IN plays a key role in IN multimerization which is essential for proviral DNA integration. Mutation of K186 of HIV-1 IN would affect the IN tetramerization and subsequently affect viral integration (Berthoux et al., 2007). For IN mutant A179P, alanine was replaced by proline but due to the cyclic binding of the three-carbon side chain to the nitrogen of the backbone, proline lacks a primary amine group ($-NH_2$) which may be required for the IN structure organization and/or for its efficient catalytic activity. Moreover, from these results, we also could not exclude the possibility that these lethal

phenotype-defective mutants could simultaneously affect to some extent IN's catalytic activities. Nevertheless, the experimental evidence derived from this study demonstrates that the C-terminal core domain-mediated chromatin binding is responsible for the lethal activity in yeast and plays an important role during viral replication.

However, how the C-terminal core domain of HIV-1 IN contributes to the chromatin binding remains to be defined. More detailed studies will be required for elucidating the mechanism(s) of the IN-involved chromatin binding. For example, we could first try to understand whether this IN-mediated chromatin binding needs other host molecule's help especially for those cellular partners of HIV-1 IN such as LEDGF/p75 and INI1.

Previous studies have shown that LEDGF/p75 is a novel cellular protein which directly interacts with HIV-1 IN and targets HIV-1 DNA integration (Cherepanov et al., 2003). It was suggested to be a significant integration cofactor for tethering viral PIC to the host chromosomes (Nishizawa et al., 2001). Moreover, LEDGF/p75-binding site on IN was also studied. One of the two regions within the central core domain of HIV-1 IN (^{161}IIGQVRDQAE^{170}) has been characterized to interact with LEDGF/p75 (Busschots et al., 2007). For the different IN mutants within this region, the interaction with LEDGF/p75 and the enzymatic activities were determined. Class II IN(I161A), IN(V165A), IN(R166A), IN(Q168A) and IN(E170A) are impaired for interaction with LEDGF/p75, but retain 3' processing and strand transfer activities (Busschots et al., 2007). 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. Interestingly, one of our

chromatin binding deficient IN mutants, V165A, located within the LEDGF/p75 binding domain ^{161}IIGQVRDQAE^{170} also showed the similar effect as Q168A during viral replication (Llano et al. 2004). Therefore, it can be speculated that interaction of LEDGF/p75 with HIV-1 IN could be involved in the IN-medicated chromatin binding. In future studies, the interaction between three chromatin-binding defective IN mutants (V165A, A179P, and KR186,7AA) and LEDGF/p75 should be further investigated.

Integrase interactor 1 (INI1) is another cellular molecule that directly interacts with HIV-1 IN and is incorporated into virion (Kalpana et al., 1994; Morozov et al., 1998). INI1 is the homologue of yeast transcription factor SNF5 and is a chromatin remodeling factor(Wang et al., 1996). As retroviral integration seems to occur preferentially in highly transcribed loci(Scherdin et al., 1990), it is tempting to speculate that the role of INI1 in the retroviral infection cycle is to target the PIC by binding IN to accessible chromatin regions, thus contributing to the bias of HIV integration for these regions of the genome. Therefore, it would be also interesting to further study the interaction between the chromatin-binding defective IN mutants (V165A, A179P, KR186,7AA) and INI1 and then to elucidate whether INI1 contributes to the IN-mediated chromatin binding.

Furthermore, previous studies have shown that multimerization of IN is important for DNA integration *in vivo* and only IN tetramer is capable of directing concerted integration(Berthoux et al., 2007). However, IN mutants located at the ^{186}KRK motif in particular changing IN lysine residue 186 to glutamate (K186Q), significantly impair IN oligomerization in the yeast two-hybrid system and decreases oligomeric forms of IN within virions (Berthoux et al., 2007). Interestingly, one of our chromatin binding

defective IN mutants KR186,7AA is also located at this motif and it should be interesting to further examine the relationship between IN multimerization and chromatin binding.

Moreover, in addition to those three chromatin binding defective IN mutants V165A, A179P and KR186,7AA , we could also perform more-detailed mutagenic analysis around the C-terminal region of IN core domain to test the effect of these new IN mutants on the chromatin binding ability. Based on the mutagenic analysis results, we would further minimize the region of HIV-1 IN for chromatin binding and design peptides or fusion proteins containing the IN chromatin binding domain to investigate whether these peptides or fusion proteins can compete with wild type IN for cellular chromatin binding. These studies may provide new information towards the development of a novel anti-IN strategy.

Importantly, this newly characterized IN activity in yeast will provide a valuable biological system for us to further study the action of HIV-1 IN during its chromatin binding, and will allow us to gain more detailed insights into the mechanisms occurring between HIV-1 IN and the mammalian system. Based on this yeast lethal phenotype system, chromatin binding ability of HIV-1 IN in yeast could be used to screen for anti-IN inhibitors that prevent the protein to associate with chromatin. All together, the definition of the critical motif(s) of IN for chromatin binding ability may serve as the basis for the discovery of new class of anti-IN agents.

Conclusions:

In this study, we have established a previously described HIV-1 IN-induced lethal phenotype system in yeast strain HP16. By using this system, we investigated the effects

of a panel of IN mutants which specifically target different regions of IN on their lethal activity in yeast.

Our results clearly revealed that, while the wild type and other IN mutants induced a lethal phenotype, three mutants(V165A, A179P, KR186,7AA) located in the C-terminal region of catalytic core domain of HIV-1 IN lost their lethal activity. Obviously, this abolishment of yeast lethality was not due to the inactivation of catalytic activity of IN, since some class I catalytic IN mutants including D64E, D116A, and D64E/D116A were still capable of inducing the lethal phenotype in yeast. We designated these three mutants as lethal phenotype defective IN mutants. Moreover, these lethality-defective mutants were introduced into HIV-1 single-cycle replication system and were further tested for their effects on HIV-1 replication. It clearly showed that none of these mutant viruses were shown to be infectious and interestingly, all of these mutant viruses could be partially complemented by a class I IN mutant (D64E). Taken together, all these data provide evidence that an unidentified IN activity contributes to its lethality in yeast and is crucial for viral replication. In attempting to explore the mechanism underlying this IN's action, our chromatin binding studies demonstrated that, in contrast to the wild type IN, all of these three lethal phenotype defective IN mutants were unable to bind to host chromatin. Overall, the C-terminal region of catalytic core domain of HIV-1 IN was first identified to bind to cellular chromatin and this chromatin binding ability of HIV-1 IN may play a critical role for its lethal phenotype in yeast and during HIV-1 infection. Further investigation of molecular basis of IN-mediated HIV DNA chromatin binding may contribute to the development of new class of anti-IN strategies.

REFERENCES

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, v. 71, p. 5871-7.

Aiken, C., and D. Trono, 1995, Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis: J Virol, v. 69, p. 5048-56.

Ansari-Lari, M. A., L. A. Donehower, and R. A. Gibbs, 1995, Analysis of human immunodeficiency virus type 1 integrase mutants: Virology, v. 213, p. 680.

Ao, Z., K. R. Fowke, E. A. Cohen, and X. 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, v. 2, p. 62.

Ao, Z., G. Huang, H. Yao, Z. Xu, M. Labine, A. W. Cochrane, and X. Yao, 2007, Interaction of human immunodeficiency virus type 1 integrase with cellular nuclear import receptor importin 7 and its impact on viral replication: J Biol Chem, v. 282, p. 13456-67.

Ao, Z., X. Yao, and E. A. Cohen, 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, v. 78, p. 3170-7.

Ariumi, Y., F. Serhan, P. Turelli, A. Telenti, and D. Trono, 2006, The integrase interactor 1 (INI1) proteins facilitate Tat-mediated human immunodeficiency virus type 1 transcription: Retrovirology, v. 3, p. 47.

Armon-Omer, A., A. Graessmann, and A. Loyter, 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, v. 336, p. 1117-28.

Barreca, M. L., K. W. Lee, A. Chimirri, and J. M. Briggs, 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, v. 84, p. 1450-63.

Berthoux, L., S. Sebastian, M. A. Muesing, and J. Luban, 2007, The role of lysine 186 in HIV-1 integrase multimerization: Virology, v. 364, p. 227-36.

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

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: Molecular Cell, v. 7, p. 1025-1035.

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, v. 70, p. 6820-5.

Bukrinsky, M., S. Haggerty, M. P. Dempsey, S. I.N., A. Adzhubei, 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, v. 365, p. 666-669.

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

Bushman, F. D., A. Engelman, I. Palmer, P. Wingfield, and R. Craigie, 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, v. 90, p. 3428-32.

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

Busschots, K., A. Voet, M. De Maeyer, J. C. Rain, S. Emiliani, R. Benarous, L. Desender, Z. Debyser, and F. Christ, 2007, Identification of the LEDGF/p75 binding site in HIV-1 integrase: J Mol Biol, v. 365, p. 1480-92.

Cai, M., R. Zheng, M. Caffrey, R. Craigie, G. M. Clore, and A. M. Gronenborn, 1997, Solution structure of the N-terminal zinc binding domain of HIV-1 integrase: Nat Struct Biol, v. 4, p. 567-77.

Calmels, C., V. R. de Soultrait, A. Caumont, C. Desjobert, A. Faure, M. Fournier, L. Tarrago-Litvak, and V. Parissi, 2004, Biochemical and random mutagenesis analysis of the region carrying the catalytic E152 amino acid of HIV-1 integrase: Nucleic Acids Res, v. 32, p. 1527-38.

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

Caumont, A. B., G. A. Jamieson, S. Pichuantes, A. T. Nguyen, S. Litvak, and C. Dupont, 1996, Expression of functional HIV-1 integrase in the yeast Saccharomyces cerevisiae leads to the emergence of a lethal phenotype: potential use for inhibitor screening: Curr Genet, v. 29, p. 503-10.

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

Chan, D. C., and P. S. Kim, 1998, HIV entry and its inhibition: Cell, v. 93, p. 681-4.

Chen, J. C., J. Krucinski, L. J. Miercke, J. S. Finer-Moore, A. H. Tang, A. D. Leavitt, and R. M. Stroud, 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, v. 97, p. 8233-8.

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

Cherepanov, P., E. Devroe, P. A. Silver, and A. Engelman, 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, v. 279, p. 48883-92.

Cherepanov, P., G. Maertens, P. Proost, B. Devreese, J. V. Beeumen, Y. Engelborghs, E. D. De Clercq, and Z. Debyser, 2003, HIV-1 integrase forms stable tetramers and associates with LEDGF/p75 protein in human cells: J. Biol. Chem., v. 278, p. 372-381.

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

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 U S A, v. 94, p. 13193-7.

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

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

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

Daniel, R., R. A. Katz, and A. M. Skalka, 1999, A role for DNA-PK in retroviral DNA integration: Science, v. 284, p. 644-7.

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, v. 294, p. 1105-8.

De Rijck, J., L. Vandekerckhove, R. Gijsbers, A. Hombrouck, J. Hendrix, J. Vercammen, Y. Engelborghs, F. Christ, and Z. Debyser, 2006, Over-expression of the LEDGF/p75 Integrase Binding Domain Inhibits HIV Replication: J Virol.

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

Derdowski, A., L. Ding, and P. Spearman, 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, v. 78, p. 1230-42.

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, v. 76, p. 12087-96.

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

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

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

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

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

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, v. 69, p. 2729-36.

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

Esposito, D.; and R. Craigie, 1999, HIV integrase structure and function: Adv Virus Res, v. 52, p. 319-33.

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

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

Fisher, A. G., M. B. Feinberg, S. F. Josephs, M. E. Harper, L. M. Marselle, G. Reyes, M. A. Gonda, A. Aldovini, C. Debouk, R. C. Gallo, and et al., 1986, The trans-

activator gene of HTLV-III is essential for virus replication: Nature, v. 320, p. 367-71.

Fletcher, T. M., 3rd, M. A. Soares, S. McPhearson, H. 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, v. 16, p. 5123-38.

Freed, E. O., 2001, HIV-1 replication: Somat Cell Mol Genet, v. 26, p. 13-33.

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 U S A, v. 94, p. 9825-30.

Gietz, D., A. St Jean, R. A. Woods, and R. H. Schiestl, 1992, Improved method for high efficiency transformation of intact yeast cells: Nucleic Acids Res, v. 20, p. 1425.

Goncalves, J., Y. Korin, J. Zack, and D. Gabuzda, 1996, Role of Vif in human immunodeficiency virus type 1 reverse transcription: J Virol, v. 70, p. 8701-9.

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, v. 299, p. 359-68.

Harrich, D., and B. Hooker, 2002, Mechanistic aspects of HIV-1 reverse transcription initiation: Rev Med Virol, v. 12, p. 31-45.

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

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 U S A, v. 91, p. 7311-5.

Hertogs, K., S. Bloor, S. D. Kemp, C. Van den Eynde, T. M. Alcorn, R. Pauwels, M. Van Houtte, S. Staszewski, V. Miller, and B. A. Larder, 2000, Phenotypic and genotypic analysis of clinical HIV-1 isolates reveals extensive protease inhibitor cross-resistance: a survey of over 6000 samples: Aids, v. 14, p. 1203-10.

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

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

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

Jenkins, T. M., A. B. Hickman, F. Dyda, R. Ghirlando, D. R. Davies, and R. Craigie, 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, v. 92, p. 6057-61.

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

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

Kalpana, G. V., A. Reicin, G. S. Cheng, M. Sorin, S. Paik, and S. P. Goff, 1999, Isolation and characterization of an oligomerization-negative mutant of HIV-1 integrase: Virology, v. 259, p. 274-85.

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

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

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

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

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

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

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

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

Limon, A., E. Devroe, R. Lu, H. Z. Ghory, P. A. Silver, and A. Engelman, 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, v. 76, p. 10598-607.

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

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

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

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

Llano, M., D. T. Saenz, A. Meehan, P. Wongthida, M. Peretz, W. H. Walker, W. Teo, and E. M. Poeschla, 2006a, An essential role for LEDGF/p75 in HIV integration: Science, v. 314, p. 461-4.

Llano, M., M. Vanegas, O. Fregoso, D. Saenz, S. Chung, M. Peretz, and E. M. Poeschla, 2004b, 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, v. 78, p. 9524-37.

Llano, M., M. Vanegas, N. Hutchins, D. Thompson, S. Delgado, and E. M. Poeschla, 2006b, Identification and characterization of the chromatin-binding domains of the HIV-1 integrase interactor LEDGF/p75: J Mol·Biol, v. 360, p. 760-73.

Lodi, P. J., J. A. Ernst, J. Kuszewski, A. B. Hickman, A. Engelman, R. Craigie, G. M. Clore, and A. M. Gronenborn, 1995, Solution structure of the DNA binding domain of HIV-1 integrase: Biochemistry, v. 34, p. 9826-33.

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

Lu, R., A. Limon, E. Devroe, P. A. Silver, P. Cherepanov, and A. Engelman, 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, v. 78, p. 12735-46.

Madani, N., and D. Kabat, 2000, Cellular and viral specificities of human immunodeficiency virus type 1 vif protein: J Virol, v. 74, p. 5982-7.

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

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, v. 278, p. 33528-39.

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

Margottin, F., S. P. Bour, H. Durand, L. Selig, S. Benichou, V. Richard, D. Thomas, K. Strebel, and R. Benarous, 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, v. 1, p. 565-74.

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

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

Masuda, T., V. Planelles, P. Krogstad, and I. S. Chen, 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, v. 69, p. 6687-96.

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

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

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

Miller, V., and B. A. Larder, 2001, Mutational patterns in the HIV genome and cross-resistance following nucleoside and nucleotide analogue drug exposure: Antivir Ther, v. 6 Suppl 3, p. 25-44.

Morozov, A., E. Yung, and G. V. Kalpana, 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, v. 95, p. 1120-5.

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

Mumberg, D., R. Muller, and M. Funk, 1994, Regulatable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use for heterologous expression: Nucleic Acids Res, v. 22, p. 5767-8.

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

Neamati, N., 2001, Structure-based HIV-1 integrase inhibitor design: a future perspective: Expert Opin Investig Drugs, v. 10, p. 281-96.

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, v. 72, p. 4104-15.

Nishizawa, Y., J. Usukura, D. P. Singh, L. T. Chylack, Jr., and T. Shinohara, 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, v. 305, p. 107-14.

Palella, F. J., Jr., K. M. Delaney, A. C. Moorman, M. O. Loveless, J. Fuhrer, G. A. Satten, D. J. Aschman, and S. D. Holmberg, 1998, Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators: N Engl J Med, v. 338, p. 853-60.

Pani, A., and M. E. Marongiu, 2000, Anti-HIV-1 integrase drugs: how far from the shelf?: Curr Pharm Des, v. 6, p. 569-84.

Parissi, V., C. Calmels, V. R. De Soultrait, A. Caumont, M. Fournier, S. Chaignepain, and S. Litvak, 2001, Functional interactions of human immunodeficiency virus type 1 integrase with human and yeast HSP60: J Virol, v. 75, p. 11344-53.

Parissi, V., A. Caumont, V. R. de Soultrait, C. Desjobert, C. Calmels, M. Fournier, G. Gourgue, M. Bonneu, L. Tarrago-Litvak, and S. Litvak, 2003, The lethal phenotype observed after HIV-1 integrase expression in yeast cells is related to DNA repair and recombination events: Gene, v. 322, p. 157-68.

Parissi, V., A. Caumont, V. Richard de Soultrait, C. H. Dupont, S. Pichuantes, and S. Litvak, 2000a, Inactivation of the SNF5 transcription factor gene abolishes the lethal phenotype induced by the expression of HIV-1 integrase in yeast: Gene, v. 247, p. 129-36.

Parissi, V., A. B. Caumont, V. R. de Soultrait, C. Calmels, S. Pichuantes, S. Litvak, and C. H. Dupont, 2000b, Selection of amino acid substitutions restoring activity of HIV-1 integrase mutated in its catalytic site using the yeast Saccharomyces cerevisiae: J Mol Biol, v. 295, p. 755-65.

Park, H. O., J. Chant, and I. Herskowitz, 1993, BUD2 encodes a GTPase-activating protein for Bud1/Rsr1 necessary for proper bud-site selection in yeast.: Nature, v. 365, p. 269-274.

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

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

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, v. 74, p. 7119-26.

Pollard, V. W., and M. H. Malim, 1998, The HIV-1 Rev protein: Annu Rev Microbiol, v. 52, p. 491-532.

Pommier, Y., C. Marchand, and N. Neamati, 2000, Retroviral integrase inhibitors year 2000: update and perspectives: Antiviral Res, v. 47, p. 139-48.

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

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

Ross, E. K., T. R. Fuerst, J. M. Orenstein, T. O'Neill, M. A. Martin, and S. Venkatesan, 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, v. 7, p. 475-83.

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

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

Scherdin, U., K. Rhodes, and M. Breindl, 1990, Transcriptionally active genome regions are preferred targets for retrovirus integration: J Virol, v. 64, p. 907-12.

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

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

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

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

Tan, W., K. Zhu, D. J. Segal, C. F. Barbas, 3rd, and S. A. Chow, 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, v. 78, p. 1301-13.

Tarrago-Litvak, L., M. L. Andreola, M. Fournier, G. A. Nevinsky, V. Parissi, V. R. de Soultrait, and S. Litvak, 2002, Inhibitors of HIV-1 reverse transcriptase and

integrase: classical and emerging therapeutical approaches: Curr Pharm Des, v. 8, p. 595-614.

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

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

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

Tsurutani, N., M. Kubo, Y. Maeda, T. Ohashi, N. Yamamoto, M. Kannagi, and T. Masuda, 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, v. 74, p. 4795-806.

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

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

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

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

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, v. 12, p. 175-85.

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

Wang, W., J. Cote, Y. Xue, S. Zhou, P. A. Khavari, S. R. Biggar, C. Muchardt, G. V. Kalpana, S. P. Goff, M. Yaniv, J. L. Workman, and G. R. Crabtree, 1996, Purification and biochemical heterogeneity of the mammalian SWI-SNF complex: Embo J, v. 15, p. 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., v. 174, p. 1477-1482.

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

Wyatt, R., and J. Sodroski, 1998, The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens: Science, v. 280, p. 1884-8.

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

Yao, X.-J., J. Lemay, N. Rougeau, M. Clement, S. Kurtz, P. Belhumeur, and E. A. Cohen, 2002, Genetic Selection of peptide inhibitors of human immunodeficiency virus type 1 (HIV-1) Vpr.: J. Biol. Chem., v. 277, p. 48816-48826.

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, v. 72, p. 4686-93.

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, v. 69, p. 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, v. 4, p. 402-10.

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

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

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, v. 101, p. 173-85.

Zhang, H., G. Dornadula, J. Orenstein, and R. J. Pomerantz, 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, v. 3, p. 165-72.

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

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

Zhu, K., C. Dobard, and S. A. Chow, 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, v. 78, p. 5045-55.

2006 Report on the global AIDS epidemic, UNAIDS, May 2006