Redirecting Lentiviral Integration: A Study of Human Immunodeficiency Virus Integrase

Jean-Philippe Belzile
Department of Human Genetics
McGill University, Montreal
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ABSTRACT

Despite great advances in our understanding of the human immunodeficiency virus (HIV) life cycle, the mechanisms that underlie the progression of HIV from cellular entry of the viral core to stable integration of the provirus are poorly understood. Sites of integration of the HIV provirus are distributed along the full length of actively transcribed genes and appear to be determined through protein-protein interactions between the viral integrase and cellular proteins.

Two cellular proteins have been proposed to perform integration targeting roles, the chromatin-remodeling factor integrase interactor 1 (INI1/hSNF5/BAF47) and the lens epithelium-derived growth factor/transcriptional co-activator (LEDGF). Here, we report the initiation of two novel integration assays to study the contribution of INI1 and LEDGF in target site selection. Elucidating these molecular determinants and their functional implications is also of particular interest to anti-HIV therapy and could have major impact on the safety of gene therapy protocols.

RESUME

En dépit des avancées majeures dans notre compréhension du cycle de vie du virus de l'immunodéficience humain (VIH), les mécanismes sous-tendant la progression de l'entrée nucléaire du cœur viral jusqu'à l'intégration stable du provirus restent obscurs. Les sites d'intégration du VIH sont distribués sur toute la longueur des gènes transcriptionnellement actifs. Des intéractions protéine-protéine entre l'intégrase virale et des protéines cellulaires semblent déterminer le choix des sites d'intégration.

Deux protéines cellulaires, le facteur de remodélage de la chromatine « integrase interactor 1 » (INI1/hSNF5/BAF47) et le facteur de transcription « lens epithelium-derived growth factor» (LEDGF), sont considérées comme les principaux candidats dans le ciblage de l'intégration. Dans ce mémoire, nous reportons l'initiation de l'élaboration de deux nouveaux modèles d'intégration, qui ont pour but l'étude de la contribution d'INI1 et de LEDGF dans le procédé de sélection des sites cibles. L'élucidation de ces déterminants moléculaires et de leurs implications fonctionnelles est particulièrement importante pour la thérapie anti-rétrovirale et pourrait avoir un impact majeur pour la biosécurité des thérapies géniques.

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ABBREVIATIONS

AD	Activation domain of GAL4
ADA	Adenine deaminase
ADA-SCID	ADA severe combined immunodeficiency
ALV	Avian leukosis virus
ASLV	Avian sarcoma leukosis virus
ASV	Avian sarcoma virus
ATR	Ataxia telangiectasia-related protein
BSA	Bovine serum albumin
CA	Capsid
CCR5	Chemokine C-C motif receptor 5
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
cPPT	Central polypurine tract
CTS	Central termination site
CXCR4	Chemokine C-X-C motif receptor 4
DBD	DNA-binding domain of GAL4
DKAs	Diketo acids
DMEM	Dulbecco's modified Eagle's medium
dNTP	Deoxynucleotide triphosphate
dsDNA	Double-stranded DNA
eIF3	Elongation initiation factor 3
Env	Envelope
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FDA	Federal Drug Agency
FIV	Feline immunodeficiency virus
FIs	Fusion inhibitors
Gag	Group-specific antigen
GFP	Green fluorescence protein
gp41	Glycoprotein 41, TM
gp120	Glycoprotein 120, SU
gp160	Glycoprotein 160, Env precursor
GST	Glutathione S-transferase
HA	Hemaglutinin
HAART	Highly active anti-retroviral therapy
HIV-1	Human immunodeficiency virus 1
HIV-2	Human immunodeficiency virus 2
HLA	Human leukocyte antigen
HMG	High mobility group
HR	Heptad repeat
HSE	Heat-shock element
HTLV-1	Human T-lymphotropic virus 1
IBD	Integrase-binding domain
IN	Integrase

INI1	Integrase interactor 1
LEDGF	Lens epithelium-derived growth factor
LM-PCR	Ligation-mediated PCR
LMO2	Lim-domain only protein 2
LTR	Long terminal repeat
MA	Matrix
MHC-I	Major histocompatibility complex class I
MLV	Murine leukemia virus
MMTV	Mouse mammary tumor virus
Mo-MLV	Moloney murine leukemia virus
MVB	Multivesicular bodies
NC	Nucleocapsid
Nef	Negative factor
NF-κB	Nuclear factor κB
NK	Natural killer cell
NLS	Nuclear localization domain
NNRTIs	Non-nucleoside reverse transcription inhibitors
NPC	Nuclear pore complex
NRTIs	Nucleoside reverse transcription inhibitors
PBS	Primer-binding site
PCR	Polymerase chain reaction
PDPs	Phenyldipyrimidines
PIC	Pre-integration complex
PIs	Protease inhibitors
Pol	Polymerase
Poly(A)	Polyadenylation
PPT	Polypurine tract
P-TEFb	Positive transcription elongation factor b
PR	Protease
R	Repeated sequence
Rev	Regulator of virion gene expression
RLU	Relative light units
RRE	Rev-responsive element
RT	Reverse transcriptase
RTC	Reverse transcription initiation complex
SCID-X1	X-linked severe combined immunodeficiency type 1
SiRNA	Small interfering RNA
SIV	Simian immunodeficiency virus
SFV	Simian foamy virus
SQLs	Styrylquinolines
SSAT	Spermine/spermidine N1-acetyl-transferase
ssRNA	Single-stranded RNA
SU	Surface glycoprotein
TAR	Transactivation responsive region
Tat	Transcriptional transactivator

TK	Thymidine kinase
TM	Transmembrane protein
U3	Sequence unique to the 3'end of the RNA
U5	Sequence unique to the 5'end of the RNA
UTR	Untranslated region
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
VSV-G	Vesicular stomatitis virus glycoprotein
WDSV	Walleye dermal sarcoma virus

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INTRODUCTION

1. RETROVIRUSES

1.1. Taxonomy of Retroviridae

Retroviruses (family of *Retroviridae*) are positive stranded ssRNA viruses that require reverse transcription as part of their replication cycle and that stably integrate their viral genomes into the host DNA. Virions are structurally complex and consist of an envelope, a nucleocapsid, a nucleoid, and a matrix protein. They have a spherical to pleomorphic shape, measure 80-100 nm in diameter, and densely dispersed glycoprotein spikes cover their surface. Originally, *Retroviridae* where divided into three subfamilies (*Oncovirinae*, *Lentivirinae*, *Spumavirinae*), but recent understanding of the structure and mechanisms of these viruses lead to their reclassification in two subfamilies: *Orthoretrovirinae* and *Spumaretrovirinae* (Table 1). *Orthoretrovirinae* are currently divided into six genera: alpharetrovirus (e.g. avian leukosis virus, ALV), betaretrovirus (mouse mammary tumor virus, MMTV), gammaretrovirus (mouse leukemia virus, MLV), deltaretrovirus (human T-lymphotropic virus 1, HTLV-1), epsilonretrovirus (Walleye dermal sarcoma virus, WDSV), and lentivirus (human immunodeficiency virus 1, HIV-1). *Spumaretrovirinae,* the other retroviral sub-family, includes one genus (spumavirus) and is typified by simian foamy virus (SFV) (1).

1.2. Genetic Organization

Three genes are shared by all species of retroviruses: *gag*, *pol* and *env.* For retroviruses of the alpha (ALV) and gamma (MLV) genera, these constitute the only viral genes (**Fig**

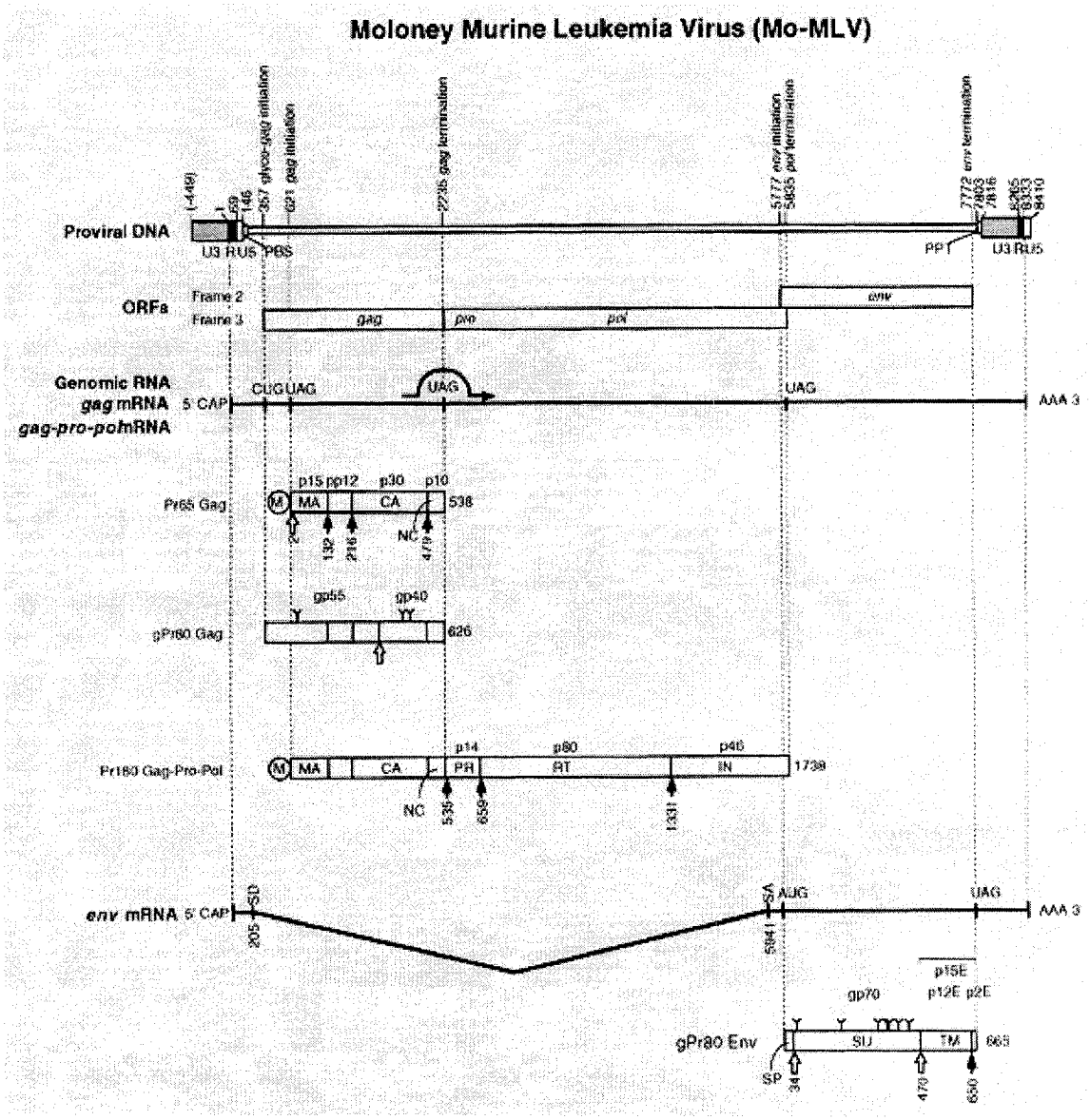

Figure 1. Schematic representation of the genome of Moloney Murine Leukemia Virus (Mo-MLV).

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1). Gag (group-specific antigen) encodes a precursor protein, which, when proteolytically processed, yields the internal structural proteins matrix (MA), capsid (CA), and nucleocapsid (NC). In some cases, such as with lentiviruses, *gag* encodes other small proteins of undefined functions. The pol gene produces a precursor polyprotein fused with the Gag precursor (Gag-Pol). Gag-Pol is also proteolytically processed and encodes for the enzymatic proteins protease (PR), reverse transcriptase (RT) and integrase (IN), which are all essential for viral replication. Retroviruses of the beta (e.g. MMTV) genera as well as simple, non-primate lentiviruses (e.g. feline immunodeficiency virus, FIV) possess an additional enzyme in the Gag-Pol precursor, a deoxyuridine triphosphatase (dUTPase). The envelope (*env)* gene encodes two subunits produced as part of a common precursor protein. The precursor is cleaved by a viral or cellular protease to yield the surface glycoprotein (SU) and the transmembrane protein (TM), which are responsible for docking the virus with its cognate cellular receptor. Deltaretroviruses (e.g. HTLV-1), epsilon-retroviruses (e.g. WDSV), spumaviruses (e.g. SFV) and complex primate lentiviruses (e.g. HIV-1) encode additional viral proteins termed "accessory proteins". Accessory proteins perform various functions in the viral replication cycle and pathogenesis. For instance, HIV-1 harbours six additional proteins: Tat (transcriptional transactivator), Rev (regulator of virion gene expression), Nef (negative factor), Vif (viral infectivity factor), Vpr (viral protein r), and Vpu (viral protein u) (**Fig. 2**). Tat, as its name implies, activates transcription of the viral genome whereas Rev is involved in viral RNA transport from the nucleus to the cytoplasm. Nef, Vif, Vpr, and Vpu modulate host immune responses and/or facilitate viral replication in specific cell types. The genes in the viral DNA (provirus) are enclosed between two identical long terminal repeats (LTR).

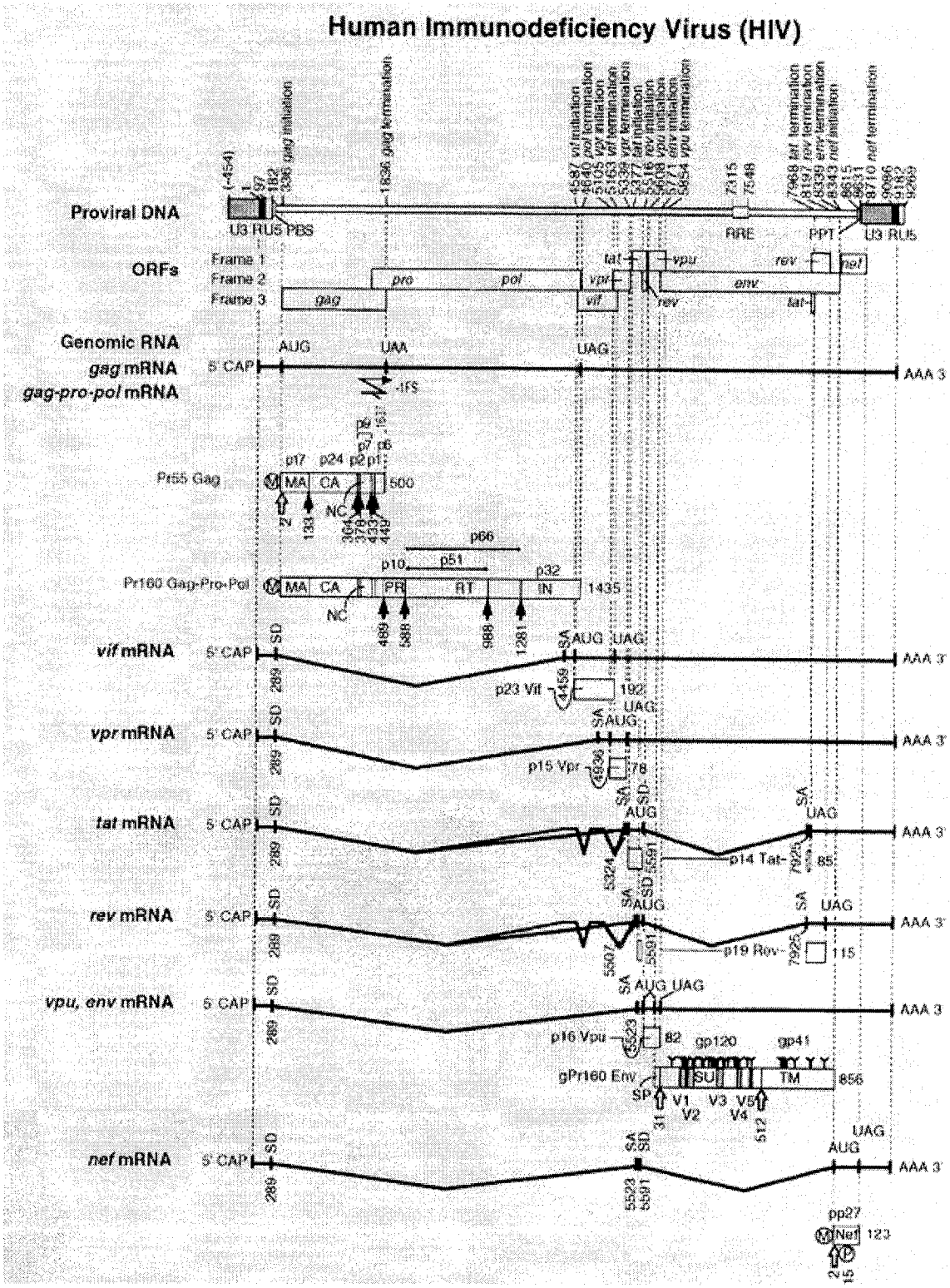

Figure 2. Schematic representation of the genome of Human Immunodeficiency Virus 1 (HIV-1).

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The LTRs can be subdivided into three regions: U3 (sequence unique to the 3'end of the RNA), R (repeated sequence) and U5 (sequence unique to the 5'end of the RNA). The transcription start site is located at the boundary of U3 and R whereas the poly(A) sequence is at the boundary of R and U5. Finally, U3 contains most of the transcriptional regulatory elements including the promoter and several enhancer regions (3).

2. RETROVIRAL REPLICATION CYCLE

HIV-1 is the most studied retrovirus and constitutes a model for the mechanism of retroviral replication (**Fig. 3**). Each of the steps in the replication cycle briefly overviewed in the current section is discussed in more details below. The retrovirus replication cycle is a highly ordered stepwise process that can be subdivided into early and late phase events. The first step of HIV replication involves binding of the Env protein to its cognate cellular receptor, CD4. This interaction leads to conformational changes effectively promoting the fusion of the virus to the cellular plasma membrane. The following steps in the replication cycle are composed of a series of poorly understood events underlying the release of the viral genomic core by a process termed uncoating and subsequently the formation of a complex competent for the initiation of reverse transcription. Then, the viral enzyme reverse transcriptase converts the viral genomic RNA to double-stranded DNA (provirus) in a complicated process involving multiple priming steps. Cellular and viral proteins bind to the newly transcribed provirus to form the pre-integration complex, which is imported in the nucleus by a poorly defined mechanism. The viral integrase subsequently catalyzes the integration of the provirus into the host genome. The late phase of the infection encompasses the expression of the

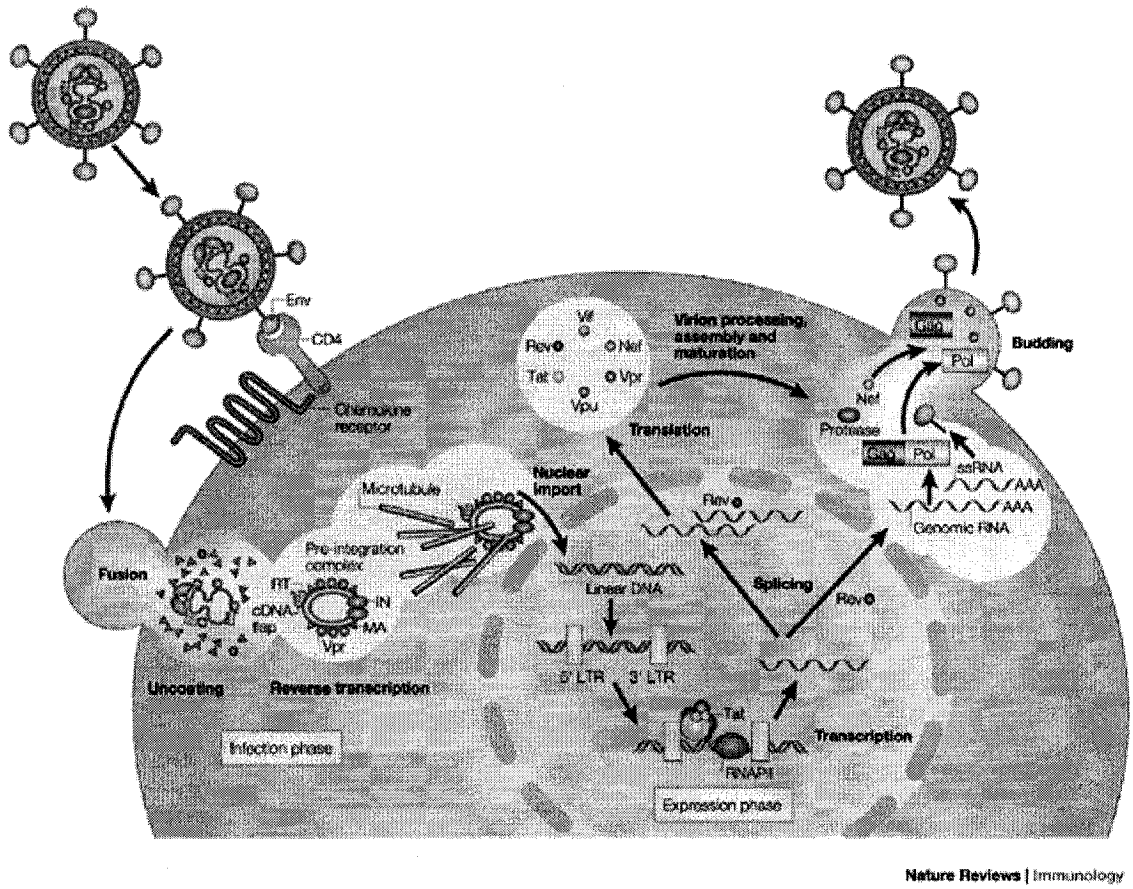

Figure 3. The life cycle of HIV.

Reproduced with permission from Nature Reviews Immunology (B.M.Peterlin, D.Trono. Hide, shield and strike back: how HIV-infected cells avoid immune eradication. Nat.Rev.Immunol., 3, 2003: 97-107, Fig 2) (4) copyright (2003) Macmillan Magazines Ltd.

viral genomic RNA as well as the expression and translation of viral essential and accessory proteins through differential splicing. The precursor structural proteins Gag accumulate at specific loci of the cellular membrane and constitute the sites at which the viral Env, genomic RNA, Gag-Pol precursor proteins, and accessory proteins are incorporated into the nascent virions. Finally, after the completion of viral assembly, the virus is released from the host cell membrane by a budding mechanism. During or shortly after budding, the viral protease cleaves the individual constituents of the precursor proteins Gag and Gag-Pol, resulting in a series of structural reorganizations and ultimately producing a mature, replication-competent HIV-1 virion (5;6).

2.1. Receptor docking and membrane fusion

The first step in HIV replication is characterized by the interaction between the envelope gp120 subunit on the virion and the cellular CD4 receptor (7;8). This interaction triggers a conformational change in gp120, now exposing the V3 loop (9-11), which enables gp120 to bind to its co-receptors, the chemokine receptors CXCR4 (12) or CCR5 (reviewed in (13)). This conformational change is thought to require the activity of a cellular surface enzyme, termed protein disulfide isomerase, which catalyses thiol/disulfide exchange by oxido-reduction (14;15). As shown by the recent crystal structure resolution of an unligated SIV gp120 protein, the most likely cystein pair candidate to promote this extensive structural remodeling would be in the V1/V2 stem (16). Indeed, CD4-bound SIV gp120 displays a 40 Å shift in the position of the V1/V2 stem compared to unligated gp120. This extensive conformation shift would liberate the other envelope subunit, gp41, from the gp120-gp41 trimers and allow gp41 to perform its role in later steps of the fusion process (16). Following binding of gp120 V3 loop to its

co-receptor, a conformational change is induced in gp41. This leads to the formation of a pre-fusion intermediate called the extended coiled-coil, which brings the N-terminal fusion peptide of gp41 close to the cell membrane, followed by its insertion into the membrane (17;18). The insertion of the N-terminal fusion peptide was shown to cause local destabilization of the membrane (17;18), and induces a second conformational change in gp41, creating a structure called the six-helix bundle (19;20). To produce this terminally stable structure, the C and N terminal heptad repeats come into tight association, while the fusion peptide and the trans-membrane domain are juxtaposed, thus bringing the viral and cellular membranes in close proximity of each other (19;20). Recent findings show that it is the transition from extended coiled-coil to six-helix bundle that promotes the merger of the two membranes and not the terminal six-helix bundle structure itself (21). Therefore, the large amount of free energy liberated during the conformational change would promote the energetically unfavorable process of fusion pore formation, thus effectively releasing the viral core in the cellular cytoplasm (21).

Genetically simpler retroviruses use a similar fusion process that appears to involve solely a primary receptor and no coreceptors. Related species of retroviruses use only a limited array of primary receptors. For instance, ecotropic MLV uses the cationic amino acid transporter of mice (mCAT-1), amphotropic MLV uses the sodium-dependent phosphate symporters Pit1 and Pit2, all species of avian leukosis viruses use either Tv-A or Tv-B, and MMTV uses transferrin receptor 1 (TRFR1) (reviewed in (22)).

2.2. Uncoating

The uncoating process is one of the least understood aspects of HIV-1 replication but recent findings might finally elucidate some aspects of the mechanisms and regulation. After its release into the cytoplasm, the viral core undergoes a progressive and extensive reduction of its protein and lipid content leading to the "uncoating" of the viral genomic RNA. It appears that the viral core is composed of a high number of weakly interacting capsid proteins (CA), of which 70% are free inside the virions (23-25). Thus, a high concentration of capsid in the virion would be required to maintain the integrity of the core. After fusion with the plasma membrane the release of free capsid proteins in the cytoplasm may promote the gradual disassembly of the core (26). This process might however require the activity of cellular factors present in activated but not in quiescent T-lymphocytes(27).

2.3. Reverse transcription

The goal of the uncoating process is to promote the formation of reverse transcription initiation complexes (RTCs), which are competent to initiate, assist, and complete reverse transcription. RTCs are large nucleoprotein structures of variable shape, consisting of packed filaments 6 nm thick with a sedimentation velocity of approximately 350 S. Vpr and IN are associated with RTCs at discrete loci. In addition, the viral genomic RNA at the center of the RTC is coated with small proteins, that are not NC (28;29). The viral RNA forms distinct and ordered loops among which the primer-binding site (PBS) and A-rich loop are central to reverse transcription initiation. The TAR (transactivation-responsive region) motif, important for high level of viral transcription in later stages of the infection, also appears to participate in the initiation of reverse transcription by either

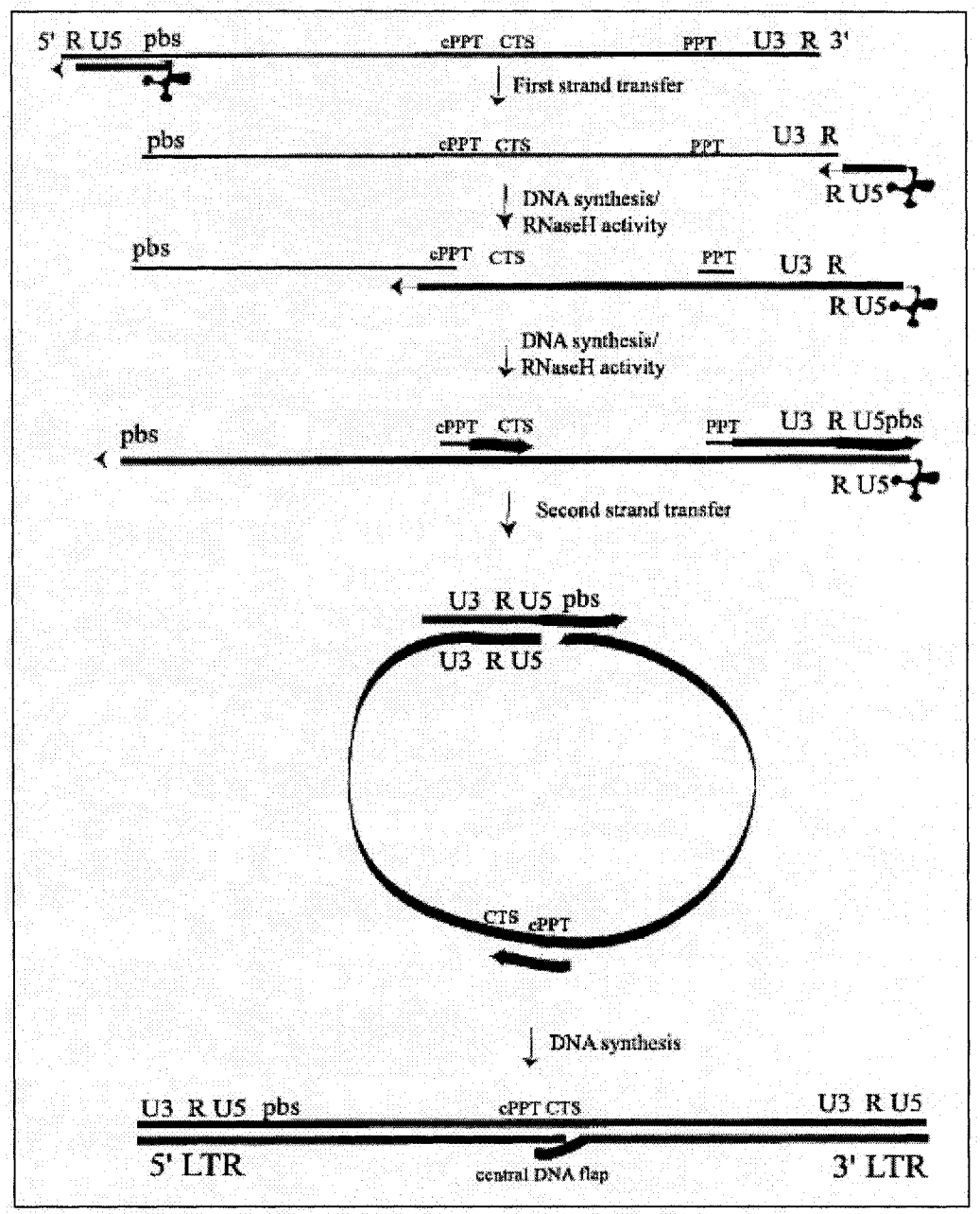

Figure 4. Mechanisms of reverse transcription.

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stabilizing directly RT or by recruiting factors that would stabilize RT(30-33). RT is the viral enzyme that catalyses the elongation of the dNTP chain onto the RNA template, ultimately converting the ssRNA viral genome to dsDNA. It is composed of a heterodimer of p66 and p51, both of which are expressed and processed as part of the Gag-Pol precursor (34).

Reverse transcription proceeds through a series of intricate steps that use *cis*-elements present on the viral genome (**Fig. 4**). HIV-1 reverse transcription is initiated by NC-mediated annealing of the $tRNA^{Lys3}$ primer to PBS. The A-rich loop appears to directly stabilize this complex by interacting with the TψC loop of the tRNA primer(30;34). Different tRNA primers are specifically used by retroviruses: $tRNA^{trp}$ for the avian sarcoma/leukosis viruses, $tRNA^{pro}$ for gammaretroviruses, $tRNA^{his}$ for fish retroviruses, $tRNA^{lys1}$ or $tRNA^{lys2}$ for spumaviruses, and $tRNA^{lys3}$ for MMTV, SIV, and HIV-1(34;35). After primer annealing, DNA synthesis proceeds up to the 5'end of the viral RNA, generating a short DNA/RNA hybrid called minus-strand strong stop DNA. The RNA moiety of the newly synthesized DNA/RNA hybrid is then degraded by the RNase H activity of p66. The minus-strand strong stop DNA then annealed to 3'end of the viral RNA by a strand transfer mechanism that uses the small homologous "R" region. Minus-strand synthesis proceeds until the 5'end of the truncated viral RNA using the minus-strand strong stop DNA as a primer. The viral RNA is concurrently degraded except for two small RNA primers known as the polypurine tract (PPT) and the central polypurine tract (cPPT). Plus-strand synthesis proceeds from PPT up to the tRNA primer at the 3'end as well as from cPPT up to a termination site known as central termination site

(CTS). The tRNA primer is subsequently degraded by the Rnase H activity of RT, allowing a second strand transfer reaction to occur using the homologous PBS at the 3'end of the negative strand. Plus-strand synthesis finally proceeds from the plus-strand cPPT priming site up to the end of the circular negative strand intermediate, displacing and duplicating the U3-R-U5 region (LTR). Plus-strand synthesis also occurs from the 5'end of the plus strand (PBS priming site) up to the CTS, thus displacing a fragment of approximately 100 bp of plus-strand (from cPPT to CTS), forming an overlapping structure known as central DNA flap (5;6;34). The role of the DNA flap in HIV nuclear import will be discussed in the next section. Other retroviruses such as spumaviruses and fish retroviruses also possess a cPPT, inducing the creation of a gap of unknown function on the plus strand. The remaining retroviruses, consisting of most of the *retroviridae*, do not possess such a central PPT. In theses cases, synthesis of the plus strand is continuous and do not result in the creation of any special DNA features (26;34;36-38).

2.4. Cytoplasmic transport and nuclear import

After cellular entry, HIV and other retroviruses had to evolve mechanisms to migrate to the nuclear periphery in the crowded, diffusion-restricted cytoplasm, and to pass the barrier of the nuclear membrane in order to finally gain access to the host genome. As uncoating and reverse transcription proceed to transform the viral core into the pre-integration complex (PIC), a structure competent for nuclear import and integration, the provirus migrates from the cytoplasmic membrane to the perinuclear region using the cellular microtubule machinery (39). Initial movement of the RTC in the cytoplasm membrane periphery occurs through the actin microfilament network (cytoskeleton). HIV-1 RTCs were shown to bind rapidly to the host cycloskeleton after infection,

possibly through a direct interaction between MA and actin (40). In addition, the viral protein Nef is essential for cytoskeleton remodeling, allowing the RTC to cross the cortical actin network (41). Moreover, an association between the RTC and the cytoskeleton is paramount for efficient reverse transcription (40). Subsequently, transport of the RTC is transferred to microtubules and proceeds towards the microtubule-organizing center. Although the interaction basis of this process has not been determined yet, this presumably involves cellular dynein-dependent motor complexes (42). A likely candidate for a structural mediator is the viral integrase, which was reported to interact with yeast microtubule-associated proteins (43).

After reverse transcription is completed, the viral genomic core is fully competent for integration and is from this point on referred to as the pre-integration complex (PIC). The PIC forms a large macromolecular structure of 400-900nm in length and 28-100 nm in diameter (reviewed in (44)). Only a few proteins have been definitely shown to be associated with PIC: the viral proteins Matrix (MA), Nucleocapsid (NC), Reverse Transcriptase (RT), IN, and Viral Protein R (Vpr) and the cellular factors Barrier to Auto-Integration Factor (BAF), High Mobility Group I/Y (HMG I/Y), and LEDGF (reviewed in (44;45))(46). A DNase-protection study showed that late PICs (10 hours after infection) had 90% of their cDNA protected whereas early PICs (8.5 hours after infection) had proteins bound only to the two LTRs. Only late PICs were competent for integration *in vitro* (45).

Most retroviruses require the breakdown of the nuclear membrane to gain access to the host genome and consequently can only infect cells that are actively dividing.

Lentiviruses and spumaviruses have however developed active nuclear import mechanisms to be proficient at infecting quiescent and terminally differentiated cells (36;47). The most favored model for HIV-1 nuclear import is that the PIC itself would include components with targeting signal able to recruit the cellular nuclear transport machinery (48). MA was the first protein reported to play this potential targeting function in HIV. MA possesses two weak but functional nuclear localization signal (NLS), mediating trafficking through the nuclear pore complex *via* interaction with importins alpha and beta (49-51). However, several studies have reported contrasting results about the involvement of MA (52-54). Vpr has also been implicated in the nuclear import of HIV PIC in the context of two non-exclusive mechanisms. Vpr could mediate nuclear import through an importin-independent pathway (55) or through an importin alpha/beta pathway (56-58). The later model relies on the direct interaction between Vpr basic NLS and importin alpha (57;59). The role of Vpr in nuclear import is however not essential, since Vpr-deficient HIV strains can replicate in non-dividing macrophages (60;61). The last viral protein candidate to mediate the import of PIC is the integrase. Through interaction with importins alpha, beta and 7 (62-64), integrase could target other proteins to the nucleus, possibly with the help of an unusual NLS signal in the central catalytic domain of IN (62;64-66). The functionality of this NLS on the integrase was however disputed in other reports (67-69). Recently, an integrase-interacting cellular transcription factor, LEDGF, was also proposed to play a karyophilic function in the HIV life cycle (46;70), despite the fact that infection in LEDGF-depleted cells did not result in impaired nuclear import (46;71). Finally, the last PIC component thought to be involved in nuclear import is a structural constituent of the provirus: the central DNA flap. It was found to dramatically increase the efficiency of nuclear import, by presumably providing

the optimal conformation of PIC for its translocation through the nuclear pore (72-74). Again, other reports questioned these results and found that the effect of the DNA flap on nuclear import was probably strain-specific (67;75).

Despite the apparent controversies concerning the involvement of individual components of the PIC in nuclear import, it is commonly accepted that these factors and possibly others, altogether participate in this very critical step of the HIV life cycle. Therefore, their redundant roles would ensure completion of nuclear import in different cell types and at different stages of the cell cycle (39;48).

2.5. Integration

After PIC access the nucleoplasm, it migrates toward the host DNA and the viral integrase catalyses the stable integration of the provirus into the host genome. Most of this mechanism remains mysterious, but the chemical basis of integration has been well defined. The integrase is a 32 kDa protein that consists of an N-terminal zinc-finger-like domain, a catalytic core domain containing a conserved D, D(35), E motif, and a SH3 C-terminal domain. The N-terminus appears to be essential for dimerization of the enzyme whereas the C-terminus is though to be involved in binding DNA. The integrase catalyses two main chemical reactions: the end-processing and the end-joining reaction (**Fig. 5**) (76-78). The end-processing reaction consists of the removal of two nucleotides by transesterification at the 3'end of a conserved CA dinucleotide at the end of the LTR. It is believed that a Mg^{2+} atom would, through activity of the conserved D,D(35)E motif, mediate deprotonation of water to facilitate transesterification (79;80). There is also evidence that end processing is a coordinated reaction, that is, mutation in the CA

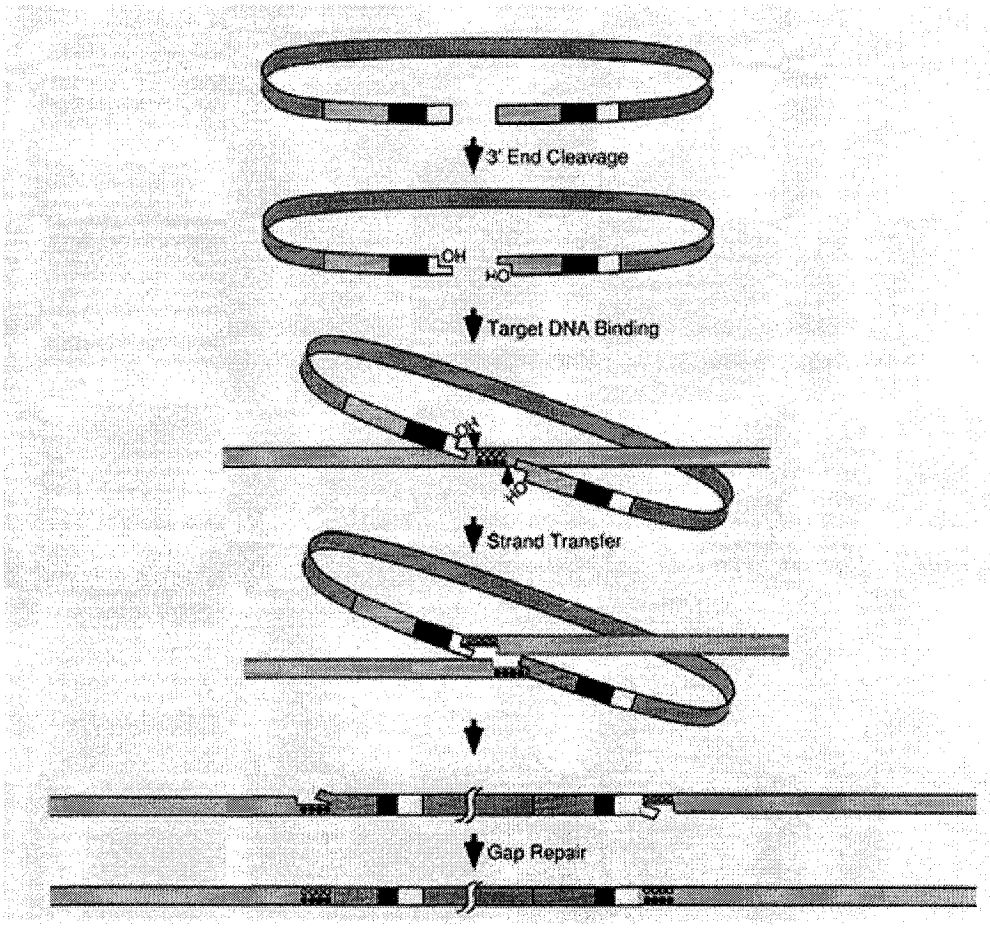

Figure 5. Schematic outline of the principal steps in retroviral DNA integration.

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dinucleotide on one LTR will also block processing at the other LTR (82). The recognition sequences of retroviral integrases on their respective LTR are relatively short and specific: 11 to 12 bp for MLV: 15 bp for ASV, and 20 bp for HIV (reviewed in (76)). The actual integration reaction *per se*, called the end-joining reaction, consists of a nucleophylic attack (simple transesterification) of the hydroxyl termini of the processed LTR ends onto a host genome target (79). As with the processing reaction, the end-joining reaction has to be coordinated for both LTR. Each LTR is inserted into the opposite strand of the host genome DNA, resulting in single-strand gaps that flank the inserted provirus. Finally, these gaps must be filled and two nucleotides from the proviral 5'overhang must be removed (76;77). This last processing step is believed to involve components of the host non-homologous end-joining DNA repair pathway and is necessary for stable integration (83;84). Although, the identities of the host factors responsible for stable integration have been highly debated, it is generally accepted that the host DNA repair machinery is involved in that process (85;86). The result of this last step in integration is a short direct duplication of the target sequence on either side of the integrated provirus. The length of this sequence duplication is characteristic of the retrovirus: 5 bp for HIV, 4 bp for Mo-MLV and 6 bp for ASV (77).

As for the choice of integration sites, it was long thought to be more or less dependent on the local DNA architecture of the target site (87). However, the recent sequencing of the human genome allowed genome-wide global analyses of integration sites for retroviruses. The main conclusion from these studies is that retroviral integration targeting might be unexpectedly similar to the well studied targeting of yeast retrotransposons (88). Sites of HIV integration seem to be concentrated in highly transcribed genes (under the control of

RNA pol II) and are distributed along the full length of these genes (89-91). Likewise, the integration of Mo-MLV is also concentrated in highly transcribed genes, but, in contrast to HIV, displays a strong bias for the early promoter regions of these genes (90;91). Avian sarcoma virus (ASV) also seems to favor transcriptionally active region of the genome, but without a particular bias towards highly transcribed regions (92;93). Recently, F. Bushman and colleagues compared the integration distribution of vectors derived from HIV, Mo-MLV, and ASLV (avian sarcoma leukosis virus) in the same cell types to eliminate possible interferences from tissue-specific expression. They found that chromosomal regions rich in expressed genes were favored for HIV integration. MLV vectors, as reported previously, showed a strong bias for transcription start sites. In contrast, ASLV vectors showed only a weak preference for active genes and no preference for transcription start regions (94). In yeast, Ty retrotransposons, which are evolutionarily related to retroviruses, use specific cellular proteins to direct their integration (reviewed in (88)). The integrase of Ty3, through its interaction with TFIIIB, typically integrates 1 to 2 bases from RNA pol III transcription start sites whereas the integrase of Ty5 integrates in heterochromatin *via* its interaction with Sir4p (reviewed in (95)). Abrogation of the interaction domains on the Ty integrase randomizes the integration spectra and modifications of these interaction domains can direct the integration to new specific sites (95). Therefore, as it is the case with yeast Ty elements, these general differences in retrovirus integration spectra suggest that interactions between components of the retroviruses pre-integration complexes and cellular proteins would actively tether the proviral integration towards defined genomic regions rather than being the results of an opportunistic access of the integrase to the most accessible chromatin-free regions of the genome (reviewed in (88;96)). This integration targeting

would provide the retrovirus a mechanism to maximize integration of the provirus at genomic loci favorable for high level of viral transcription, given that integration in sites unsuitable for high level expression can obstruct production of progeny virions (97).

2.6. Expression of the viral genome and proteins

The late phase of HIV infection begins with the expression of the viral genome and proteins from the newly integrated provirus. Efficient transcription from viral promoters of genetically simple retroviruses, such as MLV, is dependent upon the interaction of the repertoire of transcriptional elements present on its promoter and the expressed transcription factors in the infected cells. More complex retroviruses such as HIV or HTLV-1 encode their own transcription transactivator factor, providing a better control on viral expression. Therefore, contrasting with most other retroviral promoters/enhancers, the HIV LTR has only a weak basal transcriptional activity. Its promoter region comprises several transcription factor recognition elements, including nuclear factor κB (NF-κB), c-myb, stimulatory protein 1 (Sp1), and glucocorticoid receptor (GR). The HIV LTR also contains a nucleosome-binding site spanning the junction of U3 and R, the site at which transcription starts (reviewed in (98)).

HIV LTR requires the activity of the viral transactivator of transcription (Tat), which dramatically increases viral promoter activity by several hundred folds by three distinct mechanisms. Tat is a small protein of 101 amino acids that principally operate through binding to the transactivation-responsive region (TAR) on the 5'end of the leader mRNA (99;100). The first mechanism by which Tat exerts its effect on viral transcription is

through TAR-mediated recruitment of the positive transcription elongation factor b (P-TEFb) on the nascent viral mRNA (101-103). This occurs through direct interaction between Tat and cyclin T1, the cyclin partner of the P-TEFb catalytic kinase CDK9, increasing the affinity of Tat for its cognate loop-structured TAR (104). P-TEFb is one of the cellular factors that phosphorylate the C-terminal domain of RNA polymerase II, preventing transcriptional pausing and promoting polymerase processivity (105). A second TAR-dependent mechanism by which Tat can promote LTR transactivation is by recruiting histone acetyl transferase complexes to the HIV promoter. These complexes containing p300/CBP acetylate the N-terminal tails of histones in the promoter region, destabilizing histone-DNA interaction and removing nucleosomal promoter repression (106). Finally, Tat can also transactivate the viral promoter by direct, TAR-independent activation of NF-κB (107). Phosphorylation of Tat by the double-stranded RNA-dependent protein kinase PKR appears to be necessary for efficient activation of NF-κB (108).

Simpler retroviruses often have only two mRNA species: the genomic RNA and the Env-coding mRNA. However, multiple species (seven in total) of differentially spliced viral RNA are expressed from the HIV promoter and allow translation of all HIV additional proteins. As with simpler retroviruses, HIV Gag and Gag-Pol precursors are translated from the genomic RNA. The HIV Gap-Pol precursor, as with Gag-Pol precursors of most retroviruses, is translated by a rare event of ribosomal frameshifting of the Gag stop codon. Gammaretroviruses, such as Mo-MLV utilizes a different mechanism to produce the Gag-pol precursor: termination codon read-through. Both frameshifting and read-

through mechanism are rare events that typically produce one molecule of Gag-Pol for every 10 to 20 molecules of Gag. The HIV Env RNA also expresses, by leaky ribosomal scanning, the accessory protein Vpu. Each of the other HIV proteins, namely Vif, Vpr, Tat, Rev, and Nef, are expressed from unique independently, singly or fully spliced RNA species (109;110).

Effective cellular mRNA export into the cytoplasm through the nuclear pore complex is linked with the splicing machinery, which mediates the involvement of the export factor TAP/NXF1. TAP/NXF1 interacts directly with the nuclear pore complex (NPC) and can thus mediate mRNA export (111-113). Retroviruses and other nucleus-replicating viruses have developed mechanisms to promote nuclear export of unspliced or partially spliced RNA species and thus express viral proteins from these constructs (114). HIV mRNA exports relies on the virally encoded trans-acting factor Rev (115). Multimers of Rev bind unspliced or partially spliced HIV RNA on a structurally complex sequence termed Rev-responsive element (RRE), present in the nucleotide sequence of the envelop gene (116-118). A leucine motif acts as an export signal by interacting with the nuclear export factor CRM1, a protein belonging the importin/exportin family of nuclear transport receptors (119-121). This mode of nuclear-cytoplasmic transport is energy-dependent and requires Ran GTPase and components of the RAN GTPase system (111). Another complex retrovirus, HTLV-1, encodes Rex, a protein that performs essentially the same function as Rev (122). Simpler retroviruses rely on a different mechanism to export unspliced viral RNA. Their genomes harbor a special element termed constitutive transport element (CTE) (123), which can interact directly with the main cellular mRNA export factor TAP/NXF1, thus promoting efficient viral RNA transport (112;124;125).

2.7. Viral assembly, budding and maturation

HIV assembly and budding are a multi-step process involving the hijacking of cellular proteins that normally function in creating vesicles in late endosome domains called multivesicular bodies (MVB). Gammaretroviruses, alpharetroviruses, and lentiviruses normally assemble/bud from the plasma membrane but other retroviruses assemble into spherical particles in the cytoplasm before budding through the plasma membrane (126).

The construction of progeny viral particles begins with the assembly of HIV structural proteins Gag, Gag-Pol and envelope at the site of budding. It is still controversial whether assembly proceeds in a stepwise manner or concurrently, and whether the virion is constructed from pre-assembled complexes or individual proteins. Nevertheless, the assembly process can be artificially subdivided into six different mechanisms: 1) Gag-Gag interactions; 2) Gag-RNA interactions; 3) Gag-Pol interactions; 4) Gag-membrane interactions; 5) envelope incorporation; and 6) packaging of viral "accessory proteins". The first aspect of virion assembly consists of Gag multimerization, which is regarded as essential for membrane targeting (127-129). The domains of Gag responsible for these interactions include the C-terminus of CA (130;131), the p2 peptide, and the N-terminus of NC (129;132-134). Gag complexes can accumulate at the site of viral assembly through interaction of a patch of highly basic amino acids in the MA portion and the plasma membrane itself (135;136). Post-translational myristoylation of the N-terminus of the MA domain is essential for Gag membrane binding (135;137). The targeting by Gag at specific sites of the membrane is however not well understood but may involve

membrane sub-structures known as lipid rafts and possibly cell-to-cell junctions (reviewed in (138)).

Packaging of the viral genomic RNA initially requires the specific interaction between two zinc-finger motifs in the NC domain of Gag (129;132;133) and stem-loops 2 and 3 of the packaging signal of the RNA (139-141). In this respect, NC appears to be the main determinant of viral RNA encapsidation specificity (142-144). Following binding of NC to SL2-SL3, NC molecules seemingly coat the entire viral RNA in a non-specific manner (145). Interestingly, efficient assembly of Gag requires the presence of RNA (146). This is consistent with a model in which RNA allows Gag molecules to correctly align and pack (147). The Gag-Pol precursors are involved in the formation of Gag/Gag-Pol complexes, which allow transport of Gag-Pol to the site of assembly *via* the myristoylated N-terminus of the MA domain of Gag (127). Moreover, maintenance of the Gag/Gag-Pol ratio appears to be important for RNA dimerization (148), which may be important for efficient RNA packaging (149;150). Finally, Vpr and Vif are also incorporated into nascent HIV virions. Vpr is packaged through its interaction with the p6 portion of Gag (151) whereas Vif incorporation is associated with RNA packaging (152).

The precursor envelope protein, gp160, is cotranslationally inserted into the lumen of the rough ER via an N-terminal signal peptide (153;154). In the endoplasmic reticulum (ER), gp160 forms trimers through the establishment of disulfide bonds (155-157). The gp120 portion of the envelope precursor is also heavily glycosylated with high-mannose side chains. Gp160 complexes are then transported to the plasma membrane through the Golgi secretory pathway. In the Golgi, the mannose side chains on the gp120 domain are

converted to complex side chains (reviewed in (158)). In addition, gp160 is cleaved into gp41 and gp120 by a cellular protease, probably furin or a furin-like protein (159). After cleavage, gp41 associates weakly with gp120 (157). Following export to the membrane the gp41/gp120 heterotrimeric complex is anchored into the membrane by the fusion peptide at the N-terminus of gp41. The incorporation of gp120/gp41 into virions is still not well understood, but evidences suggest the involvement of MA and cellular factors. The later would account for the high efficiency at which heterologous envelope proteins can be incorporated inside HIV virions (reviewed in (5)).

After viral assembly is completed, the next step in the life cycle is the budding of the virus from the plasma membrane. As mentioned above, this event requires the recruitment of a network of cellular factors normally involved in late endosome formation and sorting. Recruitment of these cellular proteins is achieved through so-called L or Late domains on the p6 portion of the HIV gag protein. One of these domains, characterized by a P-T-A-P motif (160;161), mediates a direct interaction with the cellular protein TSG101 (162-164). TSG101 normally assists protein sorting into multivesicular bodies (MVB) (reviewed in (126;165)). Another L domain of P6, a Y-P-L-T-S-L motif, facilitate virus budding by binding to the cellular protein AIP1/Alix (166). As with TSG101, AIP1/Alix is also involved in MVB formation (167;168). Moreover, TSG101 can interact directly with AIP1, through a PTAP motif on AIP1 (166;169;170). It now appears that generation of membrane curvature during budding is accomplished through the combined actions of viral and cellular proteins, and also specialized lipids. Although this process is still not well understood, AIP1 appears to be the central mediator of membrane distortion (reviewed in (126;171)).

During or shortly after budding, protease (PR) cleaves Gag and Gag-Pol, resulting in the formation of a mature HIV virion. This constitutes the last step of the virus life cycle. PR is a member of the family of aspartic proteases and is active in a homodimeric conformation (172). PR first cleaves itself out of the Gag-Pol precursor polyproteins then sequentially and orderly cleaves the remaining viral proteins in Gag-Pol and Gag (173). Cleavage of the precursor proteins and release of individual proteins produce a major conformational rearrangement in the structure of the virion. The donut-shaped viral core is converted to an electron-dense, conical core, through realignment of CA molecules around the RNA/protein complex (147;174). Finally, it is noteworthy that proper maturation of the virus is absolutely essential for infectivity (110).

3. RETROVIRAL INFECTION AND ASSOCIATED PATHOLOGIES

3.1. Tumor viruses

Retroviruses that directly induce tumor formation as a natural step in their pathogenesis are members of the genera alpha-, beta-, gamma-, delta-, and epsilon-retroviruses. The replication capacity of the virus and the types of target cells infected both explain the impressive diversity of malignancies resulting from retroviral infection. The two main viral determinants for this diversity can be mapped to the LTR region and to the envelope gene. The viral envelope is responsible for the host and cellular tropism of the virus whereas transcriptional regulatory elements in the LTR dictate proviral expression and therefore replication capacity (175;176). Retrovirus-induced tumorigenesis can be accomplished by two distinct but intrinsically related mechanisms: 1) insertional mutagenesis of the wild type replication-competent virus (slow-transforming) and 2)

infection by a virus carrying a viral oncogene (acute-transforming) (175;176). Recombination events of LTR or viral proteins during viral replication can lead to the viral incorporation and transduction of truncated cellular proto-oncogene sequences with high transformation potential. These mutant viruses are therefore termed acute-transforming, because they produce very rapid and lethal rates of malignant development (days or weeks) (176-178). The second mechanism of retrovirus-induced tumorigenesis consists of insertional mutagenesis following infection and integration of a wild type non-acute retrovirus. Although this typically produces a latency period in the multi-step tumor formation (3-8 months), infections with non-acute retoviruses remain highly pathogenic (175;176;179;180). As its name implies, insertional mutagenesis is characterized by the disruption of a tumor suppressor gene or more likely by the activation of a cellular proto-oncogene as a result of proviral integration (175;176). Four types of integration events are generally thought to lead to oncogene activation: a) LTR-mediated transcription of cellular oncogene through promoter insertion; b) disruption of 3' transcriptional and translational suppressor sequences; c) cellular proto-oncogene truncation; and d) long-distance activation of oncogene. The latest is the most common type of activation and is the principal consequence of the effect of transcriptional enhancer elements present in the long terminal repeat of the integrated provirus(181). This activation by the proviral LTR can occur at a considerable distance from the target cellular gene. LTR activation was observed, in the case of MMTV, from provirus integrated at up to 25 kb of the target gene, in both 3' and 5' directions (179), and at up to several hundred kilobases for Mo-MLV (182).

3.2. Lentiviruses and immunodeficiency

As mentioned earlier, lentiviruses can be divided into two main categories: primate lentiviruses and genetically simpler lentiviruses. Primate lentiviruses, such as HIV-1, HIV-2 and numerous strains of SIV, primarily infect and destroy the pool of CD4-positive T-lymphocytes, inducing profound immune dysfunctions and ultimately immunodeficiency. Monocytes, dendritic cells, and other immune cells can also be directly infected by lentiviruses, through co-receptor switching (CXCR4, CCR5, and others), or indirectly, by other mechanisms, such as virion endocytosis. Infection of this expanded pool of SIV/HIV cellular target contributes to the establishment of immunodeficiency by enhancing immune dysfunctions as well as providing a covert viral reservoir (183-189). Primate lentiviruses have also evolved an array of mechanisms, to impair and evade specifically targeted immune functions in order to ensure progression of the infection from early stages to a long-term persistent and chronic infection. HIV-1, the most studied lentivirus, possesses a high degree of envelope glycosylation as well as a "last-second" mechanism of envelope unfolding that make the virus difficult to block with neutralizing antibodies. Another important HIV strategy to "fend off" immune attacks is its high mutation rate that impairs recognition of the virus by cytotoxic T lymphocytes and antibodies. Down-regulating major histocompatibility complex class I (MHC-I) molecules at the surface of infected cells and proviral promoter latency (low level of proviral expression in infected cells) also constitute two effective means that HIV employs, through its accessory proteins (the roles of which are described in the next section), to hide from the immune system (4;190). There exists a high degree of genetic divergence between all of the SIV/HIV strains, even among the same primate species,

that contributes to different receptor usage and various degrees of viral pathogenecity. The host and viral genetic requirements to produce differential viral prevalence, pathogenenesis, and resistance are however not yet understood (187).

FIV, the archetypal representative of simpler lentiviruses, appears to utilize CXCR4 has a sole cellular receptor and can infect CD4- and CD8-positive lymphocytes, macrophages and microglia. Its mechanisms of AIDS induction appears to be similar to HIV but, interestingly, FIV can selectively kill CD4-positive lymphocytes despites its concurrent infection of CD8-positive lymphocytes (191;192).

Finally, lentiviruses only rarely directly induce tumor formation, but rather, the immunodeficiency associated with their infection can lead to indirect malignant transformation, by allowing the uncontrolled proliferation of tumor-inducing viruses: Epstein-Barr virus (EBV), human papilloma virus (HPV), hepatitis C virus (HCV), hepatitis B virus (HBV), and Karposi sarcoma herpes virus (KSHV) in humans. (193).

3.3. Role of HIV accessory proteins

3.3.1. NEF

The erroneously named negative factor (Nef) is a 205-amino acid protein that is incorporated into HIV virions and cleaved by the viral protease. The major functions of Nef encompass down-regulating CD4 and MHC-I, increasing viral infectivity and modulating signaling pathway in lymphocytes and macrophages, all of which contribute to disease progression (194). Nef interacts with the cytoplasmic tail of CD4, promoting endocytosis of CD4 through a clathrin-mediated mechanism (195-197). Nef targets

internalized CD4 molecules to the lysosome for degradation, by modulating endosome-bound Beta COP-I (198). This decrease of cell-surface CD4 presumably prevents super-infection as well as suppresses T-lymphocytes immune response (199). Likewise, Nef can promote the internalization of another important immune protein, MHC-I (200). Nef specifically binds to the cytoplasmic tail of HLA-A and HLA-B MHC-I molecules, and as with CD4, enables their internalization by clathrin-mediated endosomes (201). Binding of Nef to PACS-1, reroute MHC-I-containing endosomes to the trans-Golgi network for degradation (200). The remaining cell surface MHC-I HLA-C and HLA-E molecules would prevent recognition by natural killer (NK) cells, which destroy cells expressing low levels of MHC-I molecules. Therefore, the specific down-regulation of HLA-A and HLA-B would serve as an immune evasion mechanism for the infected cells (201). Another important role of Nef in viral pathogenesis is its interference with signal transduction in infected and bystander cells. Nef interacts with several cellular signaling effectors, perturbing normal T-cell and macrophage functions, disrupting antigen-specific signaling in infected T-cells, and protecting infected T-cells against apoptosis while inducing apoptosis in bystander cells (reviewed in (202)). The last role of Nef is to increase viral infectivity through several different mechanisms: induction of components required for efficient reverse transcription (203), stimulation of lipid raft formation (204), and enrichment of cholesterol in budding virions (204). Moreover, Nef can enhance Tat-induced transcription form the viral LTR (205) as well as inhibits endocytosis of DC-SIGN, a protein involved in infection of dentrytic cells by HIV (206).

3.3.2. VIF

Vif performs four defined functions in the viral life cycle: assisting viral assembly and maturation, protecting the viral core during uncoating, increasing the effectiveness of reverse transcription, and most importantly, blocking the anti-retroviral activities of cellular factors (194). Vif also interacts with several cellular proteins, such as Sp140 nuclear protein (207), Ku70 (207), and Triad 3 (208), but the roles of these interactions in the viral life cycle have remained elusive.

Vif has been shown to contribute to viral assembly by two different mechanisms. Vif can interact directly with the viral genomic RNA, forming a 40S mRNP complex, which most likely mediates RNA-Gag interaction during assembly (209). Vif also interacts with the Gag-bound Heat-shock protein 68 (HP68), a cellular protein essential for post-translational events in immature capsid assembly (210). Several studies have also demonstrated that Vif is packaged into progeny virions at low levels (211-213), and that Vif-defective virions have abnormal cores (214). These abnormal cores appear to be more sensitive to environmental conditions, and in this respect, Vif may prevent premature degradation of the core during viral entry and uncoating (215). Vif can also increase the rate and effectiveness of reverse transcription, given that Vif mutants produce decreased RT activity *in vitro* as well as reduced synthesis of early and late DNA products (216;217). Recently, a study demonstrating Vif interaction with spermine/spermidine N1-acetyl-transferase (SSAT), an enzyme involved in polyamine metabolism, provides a possible explanation for the observed enhanced reversed

transcription in the presence of Vif. Given that polyamines are important for reverse transcription, the interaction between Vif and SSAT probably works to increase the effectiveness of this process (208). In addition to all the previously described functions of Vif in the viral life cycle, its most important role appears to be the inhibition of host restriction factors. The first identified HIV restriction factors, which Vif would inhibit is Hck tyrosine kinase. Hck tyrosine kinase negatively impacts on Vif-deficient viral production in primary lymphocytes and macrophages by a as-yet unknown mechanism. This block in replication is relieved by a direct interaction between Vif and Hck tyrosine kinase in wild type HIV virions (218). A second Vif-countered inhibitor of HIV replication was also uncovered recently (219). APOBEC3G is a cellular mRNA cytidine deaminase that, in absence of Vif, is incorporated into virions and induces guanine to adenine mutations in the plus-strand of the newly synthesized HIV provirus upon infection of a target cell (220;221). Vif can block APOBEC3G incorporation into virions by inducing the Cullin5-ubiquitination-dependent degradation of APOBEC3G (222-226) as well as inhibiting translation of ABOBEC3G (226). Some reports have also suggested that the deaminase activity of APOBEC3G does not fully account for its anti-viral function (227;228). Finally, a second member of the APOBEC family, APOBEC3F, that can restrict Vif-deficient HIV replication, was also discovered (229).

3.3.3. VPR

In addition to its contribution to reverse transcription and nuclear import of PIC, Vpr plays several other important roles in the viral pathogenesis as well as replication cycle. One of these roles is to promote G2 cell cycle arrest by blocking p34cdc2 and cdc25 (230-233). It is proposed that the direct association between Vpr and hVIP/MOV34, a

member of the elongation initiation factor 3 (eIF3) complex, would modulate eIF3 functions in G1/S and G2/M progression and cause cell cycle arrest (234;235). Moreover, there appears to be a correlation between Vpr and activation of the ATR (ataxia telangiectasia-related protein)-mediated DNA repair checkpoint (236). However, additional work is needed to show that Vpr either causes DNA damage or mimic DNA damage signals in order to activate the sensor ATR (236). Finally, protein phosphatase 2A (PP2A), a serine/threonine phosphatase involved in many aspects of cell cycle progression, had originally been implicated in Vpr-mediated arrest through direct interaction with the viral protein (237). Although the authors later retracted this previous study, observations in *S. pompe* also suggest the involvement of PP2A and Wee1 in the Vpr-induced arrest (238). The biological significance of this G2 arrest in the viral pathogenesis is still not well understood, but one possibility is that it would create favorable conditions for high-level transcription of viral genes from the LTR (239). In addition to cell cycle arrest, Vpr has been proposed to directly induce apoptosis in infected cells (240). Direct interaction between Vpr and adenine nucleotide translocator, a component of mitochondrial permeability transition pore, produces an increase of permeability of the mitochondrial membrane and subsequently a loss of the membrane potential (240). Reduced membrane potential causes the release of cytochrome c, promoting its association with other pro-apoptotic factors and ultimately inducing caspase cascades (241). The significance of Vpr-induced apoptosis in the context of other viral determinants that modulate cellular behaviors is however not well defined (232).

3.3.4. VPU

Vpu performs similar functions as Nef in AIDS pathogenesis, except that it is at the level of the endoplasmic reticulum that Vpu downregulates newly synthesized CD4 and MHC-I (242;243). Through a multiple-step mechanism that involves the gp160 envelop protein, Vpu induces degradation of CD4 molecules. In the absence of Vpu, gp160 sequesters CD4 in the endoplasmic reticulum leading to decreased numbers of CD4 molecules at the surface of the cell (244-247). The negative aspect of this system is that it reduces incorporation of gp160 in the virions and produces an accumulation of CD4 on the surface of virions, both of which interferes with viral release and infectivity (248;249). Vpu was found to promote degradation of CD4 when bound to gp160 in the ER (250), thus leading to decreased incorporation of CD4 in virions and to higher infectivity (248). This degradation mechanism involves a direct interaction between Vpu and betaTrCP, an F box protein and a component of the E3 ubiquitin ligase complex (251). This interaction leads to the polyubiquitination of CD4 and subsequently degredation by the proteasome (251-253). Vpu has also been shown to retain newly synthesized MHC-1 molecules into the ER, thus leading to downregulation of MHC molecules at the surface of the infected cells but the mechanism responsible for this activity of Vpu has not been fully investigated (254). Therefore, as in the case of Nef, Vpu contributes to immune evasion by downregulating both CD4 and MHC-1. The last contribution of Vpu to the pathogenesis of AIDS is its ability to induce apoptosis in infected cells. Through its interaction with betaTrCP, a protein involved in the proteasome-dependent degradation of multiple cellular proteins, Vpu can down-modulate the anti-apoptotic factors Bcl-xL,

A1/Bfl-1, and TRAF1, as well as increase the level of activated caspase-3 (255). The significance of Vpu-induced apoptosis on disease progression is however not understood.

3.4. Spumavirus-associated pathogenesis

The last major structurally distinct class of retrovirus consists of spumaviruses or foamy viruses. They are highly prevalent in multiple mammalian species, including primates, horse and cat. They are not normally present in human, despite several events of simian-human transmission. Spumavirus induce life-long persistent infection and display broad cellular tropisms. However, contrary to other retroviruses, they are very rarely, if not ever, associated with malignant transformation or any other pathogeneses (36).

4. RETROVIRAL VECTORS IN GENE THERAPY

4.1. Overview

The first attempts to use retroviruses as vectors to carry therapeutic genes were done in 1981. Replication competent retroviruses (also known as helper viruses) carrying HSV thymidine kinase (TK) gene were able to transform NIH 3T3 TK- fibroblast cells into TK+ cells (256;257). The major advances required to set gene therapy as promising treatment for genetic diseases came two years later. Indeed, the development of packaging cells that provide the viral proteins in *trans* and allow transduction of target cells with replication-incompetent retroviral vectors established the foundation of gene therapy (258;259). Further improvements in the packaging systems and vectors yielded "gutted" vectors, devoid of any viral protein and with the minimum viral elements necessary for integration and packaging. All other proteins were supplied *in trans* from

plasmids stably transfected in a cell line (packaging cells) (260). However, many of the original retroviral packaging cell lines often produced replication-competent helper viruses from recombination events. Later generations of packaging cells focused on an increasing numbers of split-genome packaging plasmids (Env, Gag-POL and vector are expressed from different plasmids) and incorporated minimal sequence homologies between packaging plasmids to minimize the risk of recombination events that would create replication-competent retroviruses. Another important advance in vector design was the development of pseudotyping: the ability of a particular specie of retrovirus to incorporate envelope proteins from other viral species. The most important envelope protein use in pseudotyping is the vesicular stomatitis virus G (VSV-G) glycoprotein. The cellular receptor of VSV-G is phosphatidyl serine, a lipid present on the plasma membrane of cells of most tissue and specie origins. Pseudotyping thus permit to greatly broaden or restrict the tropism of the retroviral vector. One other important application of pseudotyping is the generation of highly concentrated vectors. Indeed, VSV-G and some other viral proteins appears to reinforce the structure of retroviral vectors and permit concentration of theses vectors to very high titer ($> 10^{10}$ transduction unit/ml) by simple centrifugation (reviewed in (261-263)).

4.2. Progresses and setbacks

The first gene therapy clinical trial approved by the Federal Drug Agency (FDA) aimed at "correcting" the genetic disease ADA-SCID (adenine deaminase severe combined immunodeficiency) (264). Children suffering from ADA-SCID have a defect in the enzyme adenine deaminase and suffer from severe lack of B- and T-lymphocytes. This

first trial involved the retroviral-mediated transduction of the ADA (adenine deaminase) cDNA in large numbers of cultured-grown lymphocytes (265). Treated lymphocytes were then re-infused in patients and treatments were repeated periodically. The results of this first trial were mitigated. Partial recovery of the ADA-SCID phenotypes was observed and maintenance of low levels of ADA cDNA in the bloodstream lymphocytes was noted. However, there was no long-term maintenance of high-level expression of ADA in a significant proportion of lymphocytes, thus supporting the use of primary stem cells as a vehicle for long-term genetic correction (265).

Early vector designs used the backbone of alpha- or gamma-retroviruses because they were the best studied species. These vectors however have an intrinsic infection barrier that is very problematic for gene therapy studies: they can only access the host genomic DNA when the nuclear membrane breakdown during mitosis. Consequently, due to the lack of active PIC nuclear import, these vectors can only transduce actively dividing cells (262;263). This impediment was particularly important in cases where normally quiescent stem cells were the target of gene therapy in order to provide long-term therapeutic benefits through sustained maintenance of the therapeutic gene in the functional, differentiated progeny cells (266;267). Even under optimal culture growth conditions with cytokine stimulation, the ability of retroviral vectors to transduce stem cells, but particularly hematopoietic stem cells, remained very low (268). The immediate consequence of this reduced frequency of transduction was the limited or inexistent therapeutic benefits in most clinical trials involving re-implantation of retrovirally transduced stem cells (266;267). Levels of transduced hematopoietic stem cells (0.01% to 10%) were presumably too low to offer any therapeutic benefits (266;267).

4.3. Latest developments

More than 20 years of gene therapy research has yielded only one highly successful gene therapy trial. 14 out of 15 children afflicted with X-linked severe combined immunodeficiency type 1 (SCID-X1) where successfully treated with gene therapy (269-272). SCID-X1 is a rare lethal genetic disorder characterized by a deficiency in the common gamma chain of cytokine receptors (Gamma (c)) and resulting in the absence of mature T-lymphocyte and NK cells as well as abnormal B-cell functions. SCID-X1 is conventionally treated with moderate success rate (70-90%) by bone marrow transplantation followed by life-long immunoglobulin treatments (269;270). Cavazzana-Calvo, Fisher, and their collaborators showed that *ex vivo* retroviral vector-mediated transduction of Gamma(C) in HLA-identical bone marrow cells followed by transplantation in afflicted children were able to stably restore thymus development, T-cell functions, T-cell receptor and antibody repertoires without the need of immunoglobulin therapy (269;270). These outstanding results were however obscured by the tragic adverse cases of leukemia in three of the treated children (271-273). In the first two cases, a single vector insertion event closed to the Lim-domain only protein 2 (LMO2) leukemia-related gene followed by clonal expansion of the defective cell has caused the emergence of the lymphoproliferative syndromes (273). In the third case, the definitive sites of vector insertion remain unknown but appear to be polyclonal (272). Therefore, theses adverse events highlight the persistence and seriousness of the risks of insertional mutagenesis following retroviral vector transduction.

Three main observations can be obtained from SCID-X1 and other current gene therapy clinical trials. Firstly, the adverse development of leukemia appears to be disease-specific and transgene specific. It is proposed that the immunodeficient condition of SCID-X1 children might have contributed to the selection and rapid clonal expansion of Gamma(c)-expressing primary hematopoietic progenitors with an adverse LMO2 insertion (273). In that context, it is believed that over-expression of LMO2 and interleukin receptors could cooperate to deregulate T-cell maturation and give rise to T-cell leukemia (274;275). Secondly, further studies demonstrated that long-term hematopoietic repopulation following transplantation of transduced hematopoietic cells was derived from few clones of transduced CD34+ cells (1 to 6 clones) (276), implying that retroviral integration in LMO2 might be a common phenomenon.. Finally, the percentage of transduced CD34+ cells five years after treatment was in the range of 1 to 5%, suggesting that this frequency of transduction would have been too low to provide any therapeutic benefits in non-SCID gene therapy trials (276).

The development of lentiviral vectors based on HIV or SIV represents another milestone in the field of gene therapy (277). As mentioned above, lentiviruses have the ability to infect non-dividing or quiescent cells and could thus be used as vectors to transduce terminally differentiated cells such as neurons (277) or T-lymphocytes (278) as well as stem cells such as primary hematopoietic stem cells without the requirement of cytokine stimulation (279;280). Increased transduction capacities of hematopoietic stem cells could solve the lack of therapeutic benefits observed for gamma retroviral vectors. However, it comes at the cost of elevated numbers of vector integrated per transduced cells (average of 6 integration events per transduced cells), exacerbating the risk of

insertional mutagenesis (281;282). A number of approaches may successfully contribute to further decrease the risk of insertional mutagenesis. For instance, viral transcriptional regulatory elements are deleted from "self-inactivating" vectors to reduce the risk of transactivation of deleterious genes such as oncogenes (283-287). However, eliminating the risk of insertional mutagenesis will likely require an improved understanding of the mechanisms that control retroviral integration and, based on this understanding, the development of strategies that will permit to control vector integration and redirect it to potentially "safer" sites (88;288). Other safety issues underlying the use of lentiviral vectors in humans are related to the risk of emergence of replication-competent helper lentiviruses. This risk can be reduced but not eliminated by the use of tetracycline-inducible lentiviral vectors combined with stable packaging cell lines. Nevertheless, lentiviral vectors constitute the most promising avenue for the future of gene therapy (261;289;290).

4.4. Strategies to control vector integration

Although the adverse leukemia events in the SCID-X1 gene therapy trials appear to be disease-specific, they highlight the flaws in the current generation of vectors. Redirecting vector integration to safe genomic loci would thus constitute a powerful advancement in order to redeem the potential of gene therapy.

A strategy used by some investigators to attempt to redirect the integration consisted of fusing the HIV integrase to the DNA binding domain of other proteins in order to tether the pre-integration complex to genomic sites recognized by the fused DNA binding

domain. Integration "targeting" experiments yielded promising results *in vitro* with integrase fused to LexA (291;292) or to the zif268 zinc-finger protein (293), but were largely unsuccessful in achieving a "directed" integration in cellular models. A likely explanation is that the *in vitro* integration model lacks major cellular components such as transcription factors, transcription repressors, histones, and other cellular cofactors utilized by the integrase to direct the integration to actively transcribed regions. A more recent study showed a very specific integration pattern *in vitro* when the integrase was fused to the synthetic polydactyl zinc-finger protein E2C (294).

Recently, considerable efforts have been made by several investigators to identify the cellular effectors of lentiviral integration site selection pathway. However, depletion or abrogation of candidate proteins have failed to produce significant changes in integration frequencies or proviral expression, possibly because of functional redundancy in the integration pathway (46;295;296). Although there is evidence of integration bias with different retroviral strains, the existence of a "targeting pathway" in retroviruses has yet to be directly demonstrated.

Two cellular integrase-interacting proteins, INI1 and LEDGF, have been the main candidates studied to date. The first cellular protein that was reported to interact with IN was the integrase interactor 1 (INI1) (297). INI1, a homologue of the yeast SNF5, is a core regulatory subunit of the hSWI/SNF chromatin-remodeling complex that promotes C-MYC-mediated transactivation through hSWI/SNF (298). Although INI1 has also been shown to enhance the release of HIV particles, a generalized decrease of infectivity in INI1-depleted virions suggests a potential role for INI1 in integration (296). It has

thus been proposed that INI1 plays a role in HIV integration by permitting the remodeling of chromatin at the integration site and maintaining the proviral DNA accessible for the recruitment of transcription factors (295).

Another cellular protein that has been found to interact with IN and might influence its sites of integration is the human lens epithelium-derived growth factor/transcriptional co-activator (LEDGF) protein (299). LEDGF is known to interact with components of the general transcription machinery and promote the expression of heat-shock related genes through its interaction with heat shock elements (HSE) and stress-related elements (STRE) (300;301) . HIV IN was recently shown to form stable tetramers that associate with LEDGF (299). The full-length p75 protein, but not its p52 isoform, was indeed found to be essential for nuclear and chromosomal localization of HIV integrase (70). However, infection studies based on lentiviral vectors or wild type HIV in LEDGF-depleted cells had normal infectivity and PIC nuclear import was unaffected by LEDGF knockdown (46). This protein was nevertheless found to be a component of lentiviral PICs (46). The authors concluded that LEDGF fully accounts for cellular trafficking of diverse lentiviral integrases in simple co-localization studies but that redundancies in this essential pathway of the viral life cycle may complement the role of LEDGF during functional infections. In the same report, it was also suggested that although the overall frequency of HIV-1 integration appeared unaffected by LEDGF depletion, its role in integration site distribution merited further investigation (46). A recent report by Debyser's group contradicted previous studies and found that LEDGF depletion can significantly reduce the frequency of HIV-1 integration (71). Likewise, an integrase mutant, defective for LEDGF interaction but still catalytically active *in vitro*, was showed

to be incapable of performing integration *in vivo*. This study thus suggests that LEDGF might be the sole determinant of integration site selection (71).

5. TOOLS TO STUDY RETROVIRAL INTEGRATION

Studies of the mechanisms of integrase-driven proviral integration and integration site preferences have traditionally relied on panoply of simple but incomplete *in vitro* assays. Recent improvements in the purification methods of recombinant integrases have however led to the development of full-site concerted *in vitro* integration assays. These assays are presumably more representative of *in vivo* integration events. Likewise, a better understanding of the structure of integrases as well as concomitant improvement in cellular fluorescent microscopy and real-time quantitative PCR technology have greatly contributed to a better understanding of *in vivo* integration mechanisms and to the elucidation of integration-defective variants. The sequencing of mammalian genomes, particularly the human genome, has made possible the genome-wide analyses of the global distribution of integration sites. All of these recent developments in laboratory techniques might finally reveal poorly understood aspects of integration such as the molecular mechanisms underlying target site selection and intra-nuclear PIC trafficking.

5.1. In vitro integration assays

The most widely used *in vitro* assays to monitor the activity of mutant retroviral integrases or to test the inhibitory potential of integrase inhibitor are simple one-end integration assays. Four distinct activities of the integrase can be measured in these assays: DNA binding, 3'end processing, strand transfer, and disintegration. The latest is believed to be a relic of the *in vitro* integration conditions rather than being an actual *in*

vivo catalytic function. Nevertheless, all of these assays contribute to the study of specific residues or structural features of the integrase.

5.1.1. 3'END PROCESSING

The ability of the integrase to specifically remove two nucleotides at the 3'end of a proviral LTR can be assayed by the 3'end-processing reaction. The substrate of the reaction consists of a double-stranded synthetic oligonucleotide that contains sequences from the end of the viral LTR. In the case of HIV, the DNA substrate typically represents the 3'end of the U5 region, although sequences derived from the 5'end of U3 can also be used. The substrate is labeled at the 5'end with [γ-^{32}P]ATP and T4 polynucleotide kinase. The substrate is incubated with recombinant HIV integrase in the presence of the divalent cation Mg^{2+}. Manganese cations can also be utilized to improve the efficiency of the reaction but also increase non-specific nuclease activity. The reaction products are then resolved by denaturing-polyacrylamide electrophoresis (302).

5.1.2. ONE-END (HALF SITE) STRAND TRANSFER

Integrase-mediated integration can be monitored *in vitro* using a simple and rapid assay: the one-end strand transfer reaction. This reaction consists of the integration of one end (3'end) of a labeled oligonucleotide into a non-labeled target DNA, thus forming unresolved DNA-DNA adducts. Given that *in vivo* integration events lead to the insertion of both ends (3' and 5'ends) of the provirus, this assay therefore constitutes half of an integration reaction. The substrate of this assay is similar to the one used to measure 3'end-processing activity, but rather consists of the processed variant of this oligonucleotide. In other words, the top strand of the substrate stops immediately after

the cleavage site (the conserved CA dinucleotide). In addition, as for the 3'end-processing reaction the substrate is labeled at the 5'end with [γ -^{32}P]ATP and T4 polynucleotide kinase. The target DNA typically used in this reaction is non-labeled substrates. The reaction conditions are identical to the 3'end processing reaction except that Mn^{2+} must be used as a cofactor. Recombinant HIV integrase purified under traditional conditions (high detergent concentration) is unable to utilize their natural cofactor Mg^{2+} *in vitro*, and Mn^{2+} must thus be used to perform *in vitro* integration reactions. Integration products are then analyzed on a denaturing-polyacrylamide gel. DNA species longer than the input substrate represent integration products, whereas smaller ones are non-specific nuclease products. Finally, this assay can be modified to support high throughput screening of chemical inhibitors and has been instrumental in the identification and development of current integrase inhibitors. Briefly, the non-labeled substrate is immobilized in the wells of microtiter plates, and the reaction involves the integrase-mediated "capture" of a reporter DNA (labeled with biotin or fluorescein) (302).

5.1.3. DISINTEGRATION

The disintegration reaction is characterized by the reversible integrase-mediated removal of Y-shaped integration DNA-DNA adducts. The main advantage of this assay is that the substrate constitutes the end product of the one-end integration reaction and can thus be modified to identify important structural properties of viral and target DNA essential for integrase recognition. Given that the sequence requirements for the disintegration assay are less stringent than other integrase *in vitro* assays, the disintegration assay has also

been particularly useful for the mapping of residues important for catalytic activity. The Y-oligomer synthetic substrate is composed of oligonucleotides annealed together in order to mimic the unresolved products of a one-end integration event. One of the oligonucleotide composing the target DNA is labeled at the 5'end as described before. The reaction is performed in the same conditions as described for the 3'end-processing assay and products are resolved on a denaturing polyacrylamide gel. Disintegration is monitored by the conversion of the short oligonucleotide at the half-site integration product to the full-length target DNA (302).

5.1.4. FILTER-BINDING ASSAY

The affinity of integrase for viral and target DNA can be measured by several different methods, such as electromobility shift assay, affinity chromatography and Southwestern blotting. These techniques can be used in the mapping of the DNA-binding domain(s) of integrase or to assay the effect of potential cofactors on the affinity of integrase for its cognate substrate. A simpler and more efficient alternative is nitrocellulose filter binding. For that matter, the 5'-^{32}P-labeled U5 oligonucleotides described in the 3'end-processing assay is used. Binding of the integrase to DNA is performed for 20 minutes at 4C, in the same conditions as in the 3'end-processing assay. The reaction mixture is then passed through a nitrocellulose filter and the radioactivity retained on the filter is determined by liquid scintillation (302).

5.1.5. DETERMINATION OF INTEGRATION SITES

An important aspect of integration for gene therapy and HIV-1 disease progression is the distribution of integration sites. One means to analyze *in vitro* preferred target site, either

sequence- or structure-dependent, is to combine integration assays with PCR (polymerase chain reaction) amplification of the region flanking the integration site. For this purpose, integration assays are performed as described for one-end strand transfer reaction or as for concerted integration reaction (see below), except that the substrate is not labeled and that the target DNA is a supercoiled plasmid such as pUC18. After completion of the integration reaction, the region flanking the integration site is amplified by PCR using a ^{32}P-labeled substrate-specific primer and a second primer specific to a sequence of the plasmid. PCR products are then resolved by denaturing polyacrylamide electrophoresis. The length of a particular band designates the position of integration relative to the plasmid primer- binding site whereas the intensity of the band is indicative of the frequency of integration at a given locus (302).

5.1.6. CONCERTED INTEGRATION ASSAYS

Two-end concerted integration of a proviral sequence can be accomplished *in vitro* by two different methods. These concerted integration assays are presumably more representative of *in vivo* integration event because they involved integration of the two ends of the substrate, as it is the case *in vivo*. The first method employed to perform concerted integration is the used of purified HIV PIC from newly infected cells. This method is however time-consuming and labor-intensive, and does not readily yield data about the sequence and protein requirements (due to pleitropic effects of HIV integrase mutants on viral infectivity and PIC formation). Recently, improvements in the purification protocols for recombinant HIV integrase (303) and the use of cellular cofactors (HMG-A1 and HMG-B) (304) have lead to the development of full-site *in vitro* concerted integration assays. The main limitation of theses assays is in the difficulty to

separate concerted integration events and one-end integration products for the sequence analysis of integration sites. Different solutions to this problems have however been explored. One way to select concerted integration products for sequence analysis is to use a selection marker on the substrate followed by transformation of integration products into E. coli. The second solution promotes the use of *trans*-LTR-integrated substrates (integrase-mediated integration of two mini-viral substrates in *trans*), mimicking concerted integration of a single linear viral substrate. This way, restriction digest at two unique sites close to the U5 and U3 regions of the viral mini-substrates yield linear products that can be analyzed by PCR (304-306).

5.2. In vivo integration assays

5.2.1. STUDIES OF INTEGRATION FREQUENCY

Studies of integration frequency in infected cells are often problematic. Indeed, introduced mutations in the integrase or knockdown of a potential cellular cofactor can result in pleitropic defects in viral replication. Decreased integration frequency can thus result from specific integration defects or from defects in other steps of the viral cycle that ultimately decrease integration frequency. Several established assays are performed to determine at which step of the replication cycle the integration process is altered. The frequency of integrated viral DNA can be estimated by quantitative PCR amplification using an LTR specific primer and a non-specific *Alu*-spawning primer. Other tests include the quantification of 2-LTR circles (a marker of nuclear import), quantitative RT-PCR of the viral RNA (to monitor viral entry), and quantitative PCR determination of the amount of viral cDNA (efficiency of reverse transcription) (307).

5.2.2. ANALYSIS OF INTEGRATION SITES

Several techniques have been devised to purify and sequence genomic regions flanking integration sites. The most sensitive of these techniques is the so-called ligation-mediated PCR (LM-PCR). LM-PCR consists in the biotin-tagged, linear sequence amplification from the integrated LTR towards the undefined genomic flanking region. Paramagnetic enrichment of the newly amplified biotin-tagged fragments permits the ligation of these fragments with a synthetic adapter. PCR can then be executed to amplify the genomic fragment from the LTR to the adapter. Finally, the integration sites are sequenced directly from the PCR product or from a plasmid following cloning of the fragments. LM-PCR and other techniques used to sequence integration sites are time-consuming and labor-intensive, and thus not practically allow the sequencing of more than 50 sites (308). However, when automated, 500 to 1000 integration sites can be easily and conveniently processed by LM-PCR. Analysis of the sequenced fragments and comparison with genome and expression databank effectively identify individual integration sites and global integration site preferences (89;94).

6. HIGHLY ACTIVE ANTI-RETROVIRAL THERAPY (HAART)

6.1. Current HAART

The first effective anti-retroviral drug to be approved for HIV treatment was a nucleoside-based inhibitor of reverse transcription (nRTI) in 1987. Other compounds were later

Table 1. Anti-HIV drugs approved by the FDA.

FDA approval	Generic name	Manufacturer
	Fusion inhibitors (FIs)	
2003	Enfuvirtide (T-20)	Roche Pharmaceuticals & Trimeris
	Nucleoside reverse transcriptase inhibitors (NRTIs)	
1987	Zidovudine (AZT)	GlaxoSmithKline
1991	Didanosine (ddI)	Bristol Myers Squibb
1992	Zalcitabine (ddC)	Roche Pharmaceuticals
1994	Stavudine (d4T)	Bristol Myers Squibb
1995	Lamivudine (3TC)	GlaxoSmithKline
1997	Lamivudine+ Zidovudine	GlaxoSmithKline
1998	Abacavir	GlaxoSmithKline
2000	Abacavir + lamivudine + zidovudine	GlaxoSmithKline
2000	Didanosine (ddI)	Bristol-Myers Squibb
2001	Tenofovir disoproxil	Gilead Sciences
2003	Emtricitabine (FTC)	Gilead Sciences
2004	Abacavir+ Lamivudine	GlaxoSmithKline
2004	Emtricitabine+ Tenofovir	Gilead Sciences
	Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	
1996	Nevirapine	Boehringer Ingelheim
1997	Delavirdine (DLV)	Pfizer
1998	Efavirenz	Bristol Myers Squibb
	Protease inhibitors (PIs)	
1995	Saquinavir	Roche Pharmaceuticals
1996	Ritonavir	Abbott Laboratories
1996	Indinavir (IDV)	Merck
1997	Nelfinavir	Pfizer
1997	Saquinavir Mesylate	Roche Pharmaceuticals
1999	Amprenavir	GlaxoSmithKline
2000	Lopinavir+ Ritonavir	Abbott Laboratories
2003	Atazanavir	Bristol-Myers Squibb
2003	Fosamprenavir	GlaxoSmithKline

added to complement anti-HIV regiments and now form the backbone of highly active anti-retroviral therapy. Today, more than 20 different drugs have been approved by the FDA and target three essential steps of the HIV replication cycle (**Table 1**). NRTIs and nnRTIs (non-nucleoside reverse transcription inhibitors) aimed at blocking the activity of the viral reverse transcriptase. Protease inhibitors and the recently approved fusion inhibitor target respectively viral maturation and membrane fusion. Successful response to HAART in HIV infected patients typically result in a decrease of viral load from approximately 50 000 copies/ml prior to treatment to undetectable levels 8 to 24 weeks after initiation of HAART. However, in a significant portion of treatment-naïve patients (approximately 30%), HAART is not effective due to viral resistance, drug-drug interactions or acute drug toxicity. Likewise, patient highly experienced with HAART ultimately develop multi-drug resistance due to the emergence of resistant viral strain (through viral fitness) as well as maintenance of a viral reservoir composed of many if not all intra-host viral mutants. Therefore, new treatment strategies aim at eliminating the latent viral reservoir, counteracting drug-resistant viral strain through development of new drugs, and reducing the toxicity of currently available drugs. Promising new inhibitors aim at blocking membrane fusion, CD4 and co-receptor binding, integration and viral maturation. Other new targets, such as Tat and Vpr, are being investigated, but the developments of inhibitors are in their early stages (309;310).

6.2. Promising new drug targets

6.2.1. INTEGRASE

Several classes of small chemical inhibitors have been found to date to specifically inhibit integrase-catalyzed strand-transfer reaction. Diketo acids (DKAs) and derivatives (Merck

and Shionogi) were the first tested class of chemicals. DKAs bind at the interface of viral DNA-integrase-divalent metal ternary complex, stabilizing the 3'end-processing intermediate, and thus specifically inhibiting the strand-transfer reaction. DKAs display effectiveness in the low micromolar range, high oral bioavailability and low toxicity. Upon long exposure to the drugs, integrase can become resistant to its effects through mutations closed to the D,D(35)E catalytic residues. Currently, a napthyridine carboxamide compound (Merck) derived from the structure of DKAs displays similar strand-transfer inhibition and has shown promising results in rhesus macaques (3-4 log decrease in viral titer). It is currently undergoing early clinical trials. Two other classes of chemicals, phenyldipyrimidines (PDPs) and styrylquinolines (SQLs), have shown relatively specific inhibition of integrase and thus also constitute promising drugs for HAART. PDPs inhibit both 3'end processing and strand-transfer reactions because they block the formation of integrase-DNA complexes. In infected cells, treatment with PDPs leads to the inhibition of integration as well as an increase in 2-LTR circles. Importantly, they are active in the nanomolar range. It should however be noted that PDPs are not totally specific to the integrase since they can inhibit RT in the same concentration ranges as integrase. SQLs, like PDPs, compete with the binding of viral DNA to the integrase. Therefore, SQLs also inhibit both 3'end processing and strand-transfer reactions. SQLs have also been shown to reduce nuclear import of recombinant integrases. It is however not known whether this additional inhibitory effect of SQLs has an impact on the restriction of viral replication. Interestingly, mutant integrase resistant to SQL also display reduced viral replication. Other compounds such as guanosine quartet oligonucleotides and L-chicoric acid can potently inhibit recombinant integrase but principally affects other steps of viral replication during infection. Indeed, prolong

exposure to guanosine quartet oligonucleotides and L-chicoric acid induces the appearance of resistance mutation in gp120 rather than in the integrase. These compounds are thus not true integrase inhibitors (311).

6.2.2. VIRAL ENTRY

As mentioned above, the first viral entry inhibitor approved by the FDA is a small peptide fusion inhibitor (Enfuvirtide, Fuzeon®, Trimeris Inc and Roche Pharmaceuticals). It consists of a 36-amino acid peptide of the HR2 region of gp41. Enfuvirtide binds to the heptad repeat 1 (HR1) region of gp41, inhibiting the interaction between HR1 and HR2 and preventing the formation of the six-helix bundle. The formation of the six-helix bundle structure is necessary to initiate fusion of the viral and cellular membranes. Enfuvirtide can also interact with the stem of the V3 loop and thus inhibit CXCR4 co-receptor binding. The administration of enfuvirtide is well tolerated by patients and can result in a 10-fold reduction of viral load when combined to standard HAART regimens. The development of another promising fusion inhibitor, Tifuvirtide (Trimeris Inc.), was however halted because of formulation difficulties. Tifuvirtide is a 39-amino acid peptide inhibitor, which overlaps the sequence of enfuvirtide. It is based on a combination of sequence from HIV-1, HIV-2, and SIV (310).

Other classes of entry inhibitor comprise non-specific attachment inhibitors, and specific CD4-binding and co-receptor-binding inhibitors. Non-specific attachment inhibitors, such as dextran and heparin, block basic regions of gp120 and interfere with cell surface binding and co-receptor interaction. Another type of non-specific attachment inhibitor is Cyanovirin-N (Cellegy Pharmaceuticals). Cyanovirin-N is an 11-KDa protein derived

from a cyanobacterium specie. It interferes with CD4 and co-receptor binding of several HIV strains with different tropisms by interacting with high mannose glycoproteins on the surface of the virions. These inhibitors constitute good candidates for topical microbicide (310).

Because of generally high bioavailability, small chemical inhibitors represent the most promising candidate to block CD4-gp120 interactions. In this respect, a small chemical inhibitor (BMS 806) developed by Bristol-Myers-Squibb was originally thought to interfere with this interaction. However, later studies showed that it was rather involved in blocking the CD4-induced conformational change in gp120. BMS 806 is effective in the nanomolar range against many B clade HIV strains but is poorly or not effective against other HIV isolates. Nevertheless, good oral availability and low toxicity makes BMS 806 the first member of a new class of entry inhibitor. Other attempt at blocking the CD4-env interaction included the use anti-CD4 monoclonal antibodies and purified CD4-IgG2 fusion proteins. However, immune side effects and the need for injection might limit the use of these inhibitors. Finally, naphthalene sulfonate polymer (PRO 2000, Indevus Pharmaceuticals Inc.) has also been shown to potently inhibit HIV env-CD4 interaction and is currently in clinical trials to act as a microbicide (310).

Blocking the binding of gp120 to CCR5 represent a promising strategy to block the development AIDS since CCR5-negative individuals are highly resistant to HIV infection. Three promising candidate inhibitors are currently in development to inhibit the gp120-CCR5 interaction. A piperazine-based (SCH-D, Schering-Plough Corp.) and a spirodiketopipeparazine-based (GW 873140, GlaxoSmithKline) compound are currently

undergoing phase II clinical trials. Another chemical inhibitor, Maraviroc (Pfizer) is currently undergoing phase II/III clinical trials. Maraviroc has been showed to be active against diverse HIV isolates from different clades as well as being potent at inhibiting replication in short-term monotherapy trials (310).

Several inhibitors of CXCR4-gp120 interaction have been developed. However, toxicity, leukocytosis as well as lack of oral bioavailability have impeded progress in that field. The most effective of these inhibitors, AMD 070 (AnorMed inc.), displays good oral bioavailability and anti-viral activity, low side effects, but still produce dose-dependent increase in white blood cell counts. Long-term consequences of leukocytosis remain unknown (310).

6.2.3. MATURATION

Conventional protease inhibitors (PIs) have been highly effective in blocking HIV replication. However, prolong exposure of the drugs with the virus often leads to the development of drug-resistant strains. In that context, Panacos Pharmaceuticals developed a new kind of protease inhibitor. Rather than directly blocking the protease active site, PA 457 blocks the protease cleavage site at the junction of CA and p2. PA 457 was shown to be as effective as conventional PIs to block HIV replication *in vitro*. PA 457 is effective against conventional PI-resistant viral strains and importantly; viral escape mutants have reduced fitness. PA 457 is currently in phase II clinical trial (310).

7. OBJECTIVES OF THE CURRENT STUDY

The primary goal of this study was to initiate experimental systems that would permit the identification of the cellular proteins involved in targeting HIV-1 integration to transcriptionally active regions of the genome. Our main interest in the identification of the cellular protein involved in HIV genomic targeting is in the application of this knowledge to the development of a new class of lentiviral gene transfer vectors that would integrate in predetermined, "safer" loci of the genome. Such vectors would greatly improve the safety of gene therapy since the risk of malignancy induced by insertional mutagenesis would be greatly reduced or eliminated. In addition, identification of these cellular proteins and of their domains of interaction with viral proteins may permit the development of novel anti-HIV therapies directed at cellular rather than viral targets. Such targets would predictably be much less prone to mutations resulting in treatment resistance.

To this aim, we devised a novel *in vivo* system to study the role of candidate cellular proteins in HIV integration based on identifying the redirection of HIV-based vector integration to a specific genetic locus (the *erbB-2* gene) when normal interactions involved in vector targeting are weakened. This system will circumvent experimental limitations related to the apparent redundancy in the targeting pathway, a major impediment to the study of this pathway to this date. In addition, we will develop a novel, more complex *in vitro* model of HIV-1 integration based on dynamic chromatin templates. This model will permit to study the role of candidate cellular proteins in the integration process in presence of controlled parameters, as well as provide a more

physiologically relevant model for the screening of potential inhibitors of HIV-integration.

MATERIALS AND METHODS

CELL CULTURE AND DRUGS

293G cells (obtained from Dr. Ory, Washington University) (312) were cultured in Dulbecco's modified Eagle's medium (DMEM, GibcoBRL, Burlington, Canada) supplemented with 10% fetal bovine serum (FBS, heat-inactivated), 2 ug/ml puromycin, and 1 ug/ml doxycyclin in 5% CO2 at 37°C with constant humidity. Fresh doxycyclin was added every other day. 293GR-Luc cells were cultured in the same conditions as 293G, except that 200 ug/ml hygromycin was supplement to the growth medium. HeLa cells were cultured in DMEM supplemented with 5% FBS gentamycin (5 mg/ml) in 5% CO2 at 37°C with constant humidity. Doxycycline hyclate, puromycin dihydrochloride, and hygromycin were all obtained from Sigma-Aldrich Canada, Oakville, Ontario.

CONSTRUCTION OF LENTIVIRAL SPLIT-GENOME PACKAGING PLASMIDS

The packaging plasmids pRSV-Rev, which expresses Rev, pMDLg/pRRE, which expresses GAG-POL under Rev-post-transcriptional regulation, and the lentiviral vector pWPT-GFP, which transduces GFP (green fluorescence protein) under Rev-post-transcriptional regulation, were obtained from D. Trono (University of Geneva) (313). Rev was excised from pRSV-Rev with *HindIII* (Klenow-treated) and *XhoI* and ligated into pTRE2-Hyg (BD Biosciences, San Jose, California) at *SalI* and *EcoRV* to yield the plasmid pT2hyg-Rev, which allow the expression of Rev under tetracycline regulation.

GFP was excised from pWPT-GFP with *BamHI* and *SalI*, and replaced with a 1.6 kb luciferase coding sequence (excised from pTRE2hyg-Luc with *BamHI* and *SalI*) to yield the vector pWPT-Luc. To generate pGagPolΔIN, a packaging plasmid that expresses the Gag and Gag-Pol precursor proteins without the integrase and under the control of Rev, an *SdaI-Kpn2I* fragment from pMDLg/pRRE was first inserted into pGADT7 at the same sites. A stop codon as well as an *EcoR*I site was added at the end of the RT coding sequence by site-directed mutagenesis. The integrase was excised with *EcoRI*, the remaining Pol coding fragment was ligated on itself, and the integrase-deleted *SdaI-Kpn2I* fragment was ligated back into pMDLg/pRRE. Vpr-RT-IN fusion sequence was excised from pLR2P-VPR-RT-IN (obtained from Dr. J. Kappes, University of Alabama) (314) and ligated into a pSG5 (Stratagene, La Jolla, California) backbone (pSG5-Vpr-RT-IN) to allow expression of IN independently of Tat and packaging of IN independently of the GAG-POL precursor (315).

CONSTRUCTION OF MOLECULAR CLONES AND MUTANTS

The HIV-1 integrase cDNA (~0.9 Kb in length) was PCR-amplified from pMDLg/pRRE, adding a start codon along with an *NdeI* restriction site to the 5'end of integrase and conserving the *EcoRI* after the stop codon. IN was then ligated in pUC18 and subsequently in pGADT7 and pGBKT7 (BD Biosciences, San Jose, California) at the *NdeI* and *EcoRI* sites to yield respectively pIN, pAD-IN and pDBD-IN. pGADT7 contains the GAL4 activation domain (AD) fused to the protein of interest as well as an N-terminal hemaglutinin (HA) epitope tag and pGBKT7 contains the GAL4 DNA binding domain (DBD) fused to the protein of interest as well as a N-terminal c-myc epitope tag. All digested DNA fragments were gel purified prior to ligation.

Table 2. Oligonucleotides used in the site-directed mutagenesis of integrase.

Integrase mutants	Oligonucleotide sequence
IN (C40S, C43S)	5'-GAAATAGTAGCCAGCTCTGAT-AAATCTCAGCTAAAAGGGGAAGCCATGC-3'
IN (G47D)	5'-GTCAGCTAAAAGATGAAGCCATGCACGGA-CAAGTA-3'
IN (E48A)	5'-GCTAAAAGGGGCAGCCATGCACGGACA-AGTAGACTG-3'
IN (A49D)	5'-GCTAAAAGGGGAAGACATGCACGGACA-AGTAGACTG-3'
IN (M50E)	5'-GCTAAAAGGGGAAGCCGAGCATGGACA-AGTAGACTG-3'
IN (H51F)	5'-GCTAAAAGGGGAAGCCATGTTTGGACAAG-TAGACTG-3'
IN (G52D)	5'-GCTAAAAGGGGAAGCCATGCACGAT-CAAGTAGACTG-3'
IN (Q53L)	5'-GGGGAAGCCATGCACGGACTAGTAGAC-TGTAGCCCA-3'
IN (V54D)	5'-ATGCATGGACAAGATGACTGTAGCCCT-GGAATATGG-3'
IN (D55L)	5'-TGGACAAGTACTCTGTAGCCCTGGAATA-TGGCAGCT-3'
IN (C56K)	5'-TGGACAAGTAGACAAGAGCCCTGGAATA-TGGCAGCT-3'
IN (S57V)	5'-TGGACAAGTAGACTGTGTCCCTGGAATA-TGGCAGCT-3'
IN (P58H)	5'-TGGACAAGTAGACTGTAGCCATGGAATA-TGGCAGCT-3'
IN (G59D)	5'-TGGACAAGTAGACTGTAGCCCTGATATA-TGGCAGCT-3'
IN (I60E)	5'-AGTAGACTGTAGCCCTGGAGAATGGCAG-CTAGATTGT-3'

Integrase point mutants were built from pIN with the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California) according to the manufacturer's instructions (**Table 2**). Mutated integrase sequences were then transferred to the pGBKT7 plasmid as described above. Wild type integrase as well as the M50E mutant were also transferred to the mammalian expression plasmid pSG5. The integrase coding sequences were excised from pDBD-IN and pDBD-IN (M50E) by an *NdeI* (Klenow-treated)-*BamHI* digestion, followed by ligation in pSG5 at the *EcoRI* (Klenow treated) and *BamHI* sites to generate plasmids pSG5-IN and pSG5-IN (M50E). N-terminal integrase deletion mutants were generated by PCR with anti-sense primer 5'-GCTCCGGAATTCCATGT-3' and a second primer, adding a start codon and an *NdeI* site at the specified position (**Table 3**). C-terminal integrase deletion mutants were generated by PCR with the sense primer 5'-AGGAAACATATGTTTTTAGATGGAA-TAGAT-3' and a second primer, adding a stop codon and a *BamHI* site at the specified position (**Table 3**). Integrase deletion PCR products were digested with *EcoRI* and *NdeI*, gel purified, and finally ligated into pGBKT7 at the same restriction sites.

The INI1 cDNA (obtained from C. Wright, University of North Carolina) was extracted from a pCDNA3.1 plasmid (Invitrogen, Carlsbad, California,) with *EcoRI* and *XhoI* and ligated into the mammalian expression plasmid pCMV-HA (BD Biosciences, San Jose, California) to yields pCI1H. INI1 was then excised from pCI1H with *SfiI* and *XhoI* and ligated into pGBKT7 (pDBD-INI1) and pGADT7 (pAD-INI1). To produce INI1 fused to a N-terminal glutathione S-transferase (GST), INI1 was excised from pGADT7 with *EcoRI* (Klenow-treated) and *BamHI* and ligated into pGEX4T-1 (Amersham Biosciences, Piscataway, New Jersey) at the same sites.

Table 3. Oligonucleotides used to generate integrase truncations

Integrase truncations	Oligonucleotide sequence
IN (1-47)	5'-CATGGAATTCTTACCCTTTTAGC-TGACATTTATCACA-3'
IN (1-54)	5'-CTGGGAATTCTTATACTTGTCC-ATGCATGGCTT-3'
IN (38-288)	5'-AGGACATATGGCCAGCTGTG-ATAAATGTCAGC-3'
IN (44-288)	5'-TAAACATATGCAGCTAAAAGGG-GAAGCCAT-3'
IN (48-288)	5'-GCTACATATGGAAGCCATGCAT-GGACAAG-3'
IN (55-288)	5'-TGGACATATGGACTGTAGCCCA-GGAATATGG-3'
IN (61-288)	5'-CCCACATATGTGGCAGCTAGAT-TGTACACATTTAGAA-3'

The LEDGF cDNA was cloned from HeLa total RNA by RT-PCR, adding an *EcoRI* site 5' to the start codon and a *BamHI* site after the stop codon. The digested, gel-purified LEDGF PCR product was then ligated into pGADT7 to generate pAD-LEDGF.

CONSTRUCTION OF E2C-FUSION EXPRESSION PLASMIDS

E2C was PCR-amplified from pCDNA-KREB-E2C (obtained from C. Barbas, III, Scripps Research Institute, La Jolla, California), conserving the N-terminal nuclear localization signal (NLS) and the C-terminal HA tag. In addition, a start codon as well as a *BamHI* site was added at the 5'end and a *BglII* site was added after the stop codon. NLS-E2C-HA was then ligated into pSG5 at the *BamHI* and *BglII* sites (pSG5-E2C). Full-length INI1, INI1 (residues 183-294), full-length LEDGF, and LEDGF (residues 340-417) were PCR-amplified (**Table 4**) from pAD-INI1 and pAD-LEDGF, adding a start codon as well as an *EcoRI* site at the 5'end. At the 3'end, a *BamHI* site was added, but the stop codon was omitted. PCR products were gel purified, digested with *EcoRI* and *BamHI*, and ligated into pSG5-E2C at the same sites.

CONSTRUCTION OF SIRNA-EXPRESSION PLASMIDS

Sense and anti-sense synthetic oligonucleotides (**Table 5**) containing *XhoI*- and *XbaI*-compatible overhangs were annealed with each other and subsequently ligated into pre-digested pSuppressor plasmid (Imgenex Corporation, San Diego, California) according to the manufacturer's instructions.

Table 4. Oligonucleotides used in the construction of E2C-fusion plasmids.

Proteins		Oligonucleotide sequences
NLS-E2C-HA	*Sense*	5'-TCAAAGGATCCATGCCGAAA-AAGAAACGCAAAGT-3'
	Anti-sense	5'-GTACAGATCTTCAAGAAGC-GTAGTCCGGA-3'
INI1 (full length)	*Sense*	5'-GAACGAATTCATGATGATGAT-GGCGCTGAGCAAGACC-3'
	Anti-sense	5'-ACAGGGATCCCCAGGCCGGC-CCCGTGTT-3'
INI1 (183-294)	*Sense*	5'-GAACGAATTCATGCCCGAGG-TGCTGGTCCCC-3'
	Anti-sense	5'-ACAGGGATCCGGCAAACTTC-TCTGGTGAGTTCTCCTTC-3'
LEDGF (full length)	*Sense*	5'-GGCCGAATTCATGACTCGCGA-TTTCAAACCTGGAGAC-3'
	Anti-sense	5'-CCGCGGATCCGTTATCTAGTG-TAGAATCCTTCAGAGATATT-3
LEDGF (340-417)	*Sense*	5'-AGTTGAATTCATGGTGGAG-AAGAAGCGAGAAACAT-3'
	Anti-sense	5'-TATAGGATTCTGTAGACTT-TTCCATGATTACCTGAC-3'

Table 5. Oligonucleotides used in the construction of siRNA-expressing plasmids.

SiRNA		Oligonucleotide sequences
INI1		
SiRNA#1	*Sense*	5'-TCGATGCCGCCGCAATGATGATG-ATTTCAAGAGAATCATCATCATT-GCGGCGGCATTTTT-3'
	Anti-sense	5'-CTAGAAAAATGCCGCCGCAATG-ATGATGATTCTCTTGAAATCATC-ATCATTGCGGCGGCA-3'
SiRNA#2	*Sense*	5'-TCGACCGCAATGATGATGATGG-CGCTTTCAAGAGAAGCGCCATCA-TCATCATTGCGGTTTTT-3'
	Anti-sense	5'-CTAGAAAAACCGCAATGATGATG-ATGGCGCTTCTCTTGAAAGCGCC-ATCATCATCATTGCGG-3'
SiRNA#3	*Sense*	5'-TCGACCTGGTAACCAGCCCATCA-TTCAAGAGATGATGGGCTGGTT-ACCAGGTTTTT-3'
	Anti-sense	5'-CTAGAAAAACCTGGTAACCAGCC-CATCATCTCTTGAATGATGGGCT-GGTTACCAGG-3'
LEDGF		
SiRNA#1	*Sense*	5'-TCGACCCCGAAACATGACTCGCG-ATTTTTCAAGAGAAAATCGCGAG-TCATGTTTCGGGGTTTTT-3'
	Anti-sense	5'-CTAGAAAAACCCCGAAACATGAC-TCGCGATTTTCTCTTGAAAAATC-GCGAGTCATGTTTCGGGG-3'
SiRNA#2	*Sense*	5'-TCGACTACACTAGATAACTAGGT-TGACTTCAAGAGAGTCAACCT-AGTTATCTAGTGTAGTTTTT-3'
	Anti-sense	5'-CTAGAAAAACTACACTAGATAA-CTAGGTTGACTCTCTTGAAGTCA-ACCTAGTTATCTAGTGTAG-3'
SiRNA#3	*Sense*	5'-TCGACTAGGTTGACATACCTGG-TTCAAGAGACCAGGTATGTCAA-CCTAGTTTTT-3'
	Anti-sense	5'-CTAGAAAAACTAGGTTGACATAC-CTGGTCTCTTGAACCAGGTATGTC-AACCTAG-3'

CONSTRUCTION OF LENTIVIRAL PACKAGING CELL LINE

The packaging cell line was built from 293G cells, which express the vesicular stomatitis virus glycoprotein (VSV-G) envelope protein under the repression of tetracycline-responsive element (obtained from Dr. Ory, Washington University) (312). 40 ug of linearized pWPT-Luc and 5 ug of linearized pT2hyg-Rev (molar ratio of 5:1) were transfected into $1X10^6$ 293G cells with Lipofectin (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were selected with 200 ug/ml hygromycin (Sigma-Aldrich Canada, Oakville, Ontario) for 14 days. Single-cell clones were then grown in 96-well plates by limited dilutions. Individual clones were expanded and analyzed for their luciferase activity and Rev expression. The clone that provided the highest level of induction of Rev expression in absence of doxycycline while maintaining a minimal level of expression in its presence constituted the 293GR-Luc lentiviral vector packaging cell line. pGagPolΔIN and pSG5-Vpr-RT-IN would be supplied to the packaging cells by transient transfection.

LUCIFERASE ASSAYS

Two hundred thousand 293GR-Luc clonal cells were platted into the well of a six-well plate. Forty-eight hours later, cells were harvested and assayed for their luciferase activity using the Luciferase Reporter Assay Kit (BD Biosciences, San Jose, California) according to the manufacturer's instructions. Reaction lysates were analyzed on a luminometer in Costar white opaque 96-well plates using automatic injectors, with a one-second injection and reading delay. Relative light units were standardized with the

amount of proteins in each reaction. Proteins were quantified by the method of Lowry (Dc Protein Assay, Bio-Rad Laboratories, Hercules, California)

INDUCTION OF REV EXPRESSION

For Rev expression analysis, five hundred thousand cells from selected 293GR-Luc clones were platted into two 10-cm culture dishes with DMEM (10% FBS). Doxycycline was only added to one of the two dishes. Hygromycin and puromycin were omitted. Cells were grown for 5 days at 37°C and RNA was extracted using the RNeasy RNA extraction kit (Qiagen, Mississauga, Ontario).

YEAST TWO-HYBRID ASSAYS

The host yeast strain used for the two-hybrid experiments was the AH109 strain (Matchmaker Two-Hybrid System 3, BD Biosciences, San Jose, California), mutated for *LEU, TRP, HIS,* and *ADE (*full genotype*: MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ,. LYS2 : : GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3, GAL2*$_{UAS}$*-GAL2*$_{TATA}$*-ADE2, URA3 : : MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-lacZ).* *LEU* and *TRP* are nutritional markers for the transformation by pGADT7- and pGBKT7-derived vectors. *HIS* and *ADE* are nutritional markers for the activation of the GAL4 promoter and require the interaction between the "bait" and "library" proteins. In addition to nutritional selective markers, the AH109 strain comprised *MEL1* and *LacZ* reporter genes for colorimetric identification and quantification of interactions. Yeast transformation was performed by conventional lithium acetate-DMSO-based high efficiency protocol, using 100 ng of plasmid DNA and 100 ng of carrier sperm DNA. AH109 cells were first transformed with pAD-INI1, pAD-IN, and pAD-LEDGF to generate the parental strains A/AD-INI1, A/AD-IN, and A/AD-LEDGF. These strains were then used as a platform for transformation with DBD-

expression plasmids, including mutated integrase fused to DBD, to monitor binding affinity. A/AD-INI1 and A/AD-LEDGF two-hybrid transformants were platted on synthetic complete medium without leucine and tryptophane as well as synthetic complete medium without leucine, tryptophane, and histidine. A/AD-IN two-hybrid transformants were platted on synthetic complete medium without leucine and tryptophane, synthetic complete medium without leucine, tryptophane, and histidine, and also synthetic complete medium without leucine, tryptophane, histidine, and adenine. All two-hybrid synthetic complete plates were coated with X-alpha-gal (BD Biosciences, San Jose, California) 1 hour prior to seeding. Colonies were grown for up to ten days at 30°C. Interaction levels were scored in function of the number and growth rates of colonies as well as *MEL1* activity (intensity and time of blue color conversion)

GST PULL-DOWN

Recombinant GST and GST-IN proteins were first expressed in *E. coli* (BL21) from the pGEX-4T-1 plasmid (Amersham Biosciences) upon induction with IPTG. They were then extracted in NET-N buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris pH 8.0, 0.5% NP-40, 1uM pepstatin, 2uM leupeptin, 30 nM aprotinin, and 0.1 mM PMSF) by sonication, incubated on glutathione-sepharose 4B beads (Amersham Biosciences, Piscataway, New Jersey) for 1 hour at 4°C, and washed three times with NET-N buffer. Bead-bound GST and GST-INI1 extracts were then resolved on a 10% denaturing polyacrylamide gel and quantified on gel using bovine serum albumin standards. Wild type and mutant integrases were transcribed and translated *in vitro* from pGBKT7- and pSG5-based plasmids in the presence of ^{35}S-methionine using rabbit reticulocyte lysate (TnT, Promega, Madison, Wyoming) according to the manufacturer's instructions. Ten

ul of ^{35}S-labeled products were incubated with 20 ug of bead-bound GST-INI1 or GST (control) for 2 hours at 4°C in GST-binding buffer (20 mM Hepes pH 7.5, 150 mM KCl, 0.1% NP-40, 10 mM $MgCl_2$, 5% glycerol, 1 mM DTT, 0.1 mg/ml BSA, 1uM pepstatin, 2uM leupeptin, 30 nM aprotinin, and 0.1 mM PMSF). Bead-bound proteins were centrifuged at 500 X g for 3 minutes, washed twice with GST-binding buffer, washed twice with GST-binding buffer without BSA, resuspended in 20 ul of protein loading buffer, and resolved on a 10% SDS polyacrylamide gel. Interacting ^{35}S-labeled IN mutants were finally visualized by autoradiography (at -80°C) using sodium salicylate as an amplifier. Comparison of binding affinity was performed with the help of computer-assisted densitometry.

SEMI-QUANTITATIVE RT-PCR

The level of Rev expression in selected 293GR-Luc clones was determined by semi-quantitative RT-PCR. 500 ng of RNA were reverse transcribed in 20 ul of RT reaction buffer (50 mM Tris pH 8.3, 75 nM KCl, 3 mM $MgCl_2$, 25 ng/ul poly(dT) oligonucleotides, 10 mM DTT, 1U/ul Rnase inhibitors, 125 uM dNTPs and 10U/ul of Mo-MLV RT) (Invitrogen, Carlsbad, California) for 50 min at 37°C. Two ul of reverse transcribed products were amplified by PCR in a 50-ul reaction using the sense primer 5'-acctcctcaaggcagtcaga-3' and the anti-sense primer 5'-tcccagaagttccacaatcc-3'. The PCR reaction included 20 mM Tris pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 1 uM of primers, 0.1 mM dNTPs, and 0.03 U/ul *Taq* polymerase. The PCR conditions were 15 min at 95°, followed by 34 cycles of [30 sec at 95°C, 1 min at 57°C, 1min15sec at 72°C], and finally 5 min at 72°C. PCR products in the non-saturated linear amplification range were

resolved on 2% agarose gel electrophoresis and visualized with ethidium bromide. Band intensity was determined by computer-assisted densitometry. Rev PCR amplifications were standardized with the amplification of the endogenous histone 3.3 RNA with sense primer 5'-gtaaagcacccaggaagcaa-3' and anti-sense primer 5'-acgctggaagggaagtttg-3'. PCR conditions were the same as for Rev except that the concentration of $MgCl_2$ in the PCR buffer was 3 mM and that the number of PCR cycles was 35.

RESULTS

CONCEPTION OF THE *IN VIVO* TARGETING SYSTEM

Our *in vivo* integration model to study integration targeting mechanism relies on the over-expression of full-length and truncated INI1 or LEDGF in target and lentiviral vector-producing cells (**Fig. 6**). In this system, INI1 and LEDGF were fused to the hexadactyl synthetic zing-finger protein E2C, which recognizes a unique 18-bp sequence in the 5'untranslated region of the gene *ErbB-2* on chromosome 17 (316;317). In the case of INI1, *in vivo* integration performed in presence of the integrase binding domain (IBD, minimal binding domain) of INI1 fused to E2C (IBD(INI1)-E2C) and with siRNA-mediated suppressed endogenous INI1 expression is expected to "target" vector integration, at least to a certain extent, to the vicinity of *erbB-2* given the known interaction between IBD(INI1) and IN. Irrespective of whether or not INI1 is normally involved in HIV integration targeting, we should observe enrichment of integration at *ErB-b2* with the fusion of IBD (INI1) to E2C, which creates a protein with specific DNA-binding activity. The INI1(full length)-E2C integration experiments will clearly determine the potential role of INI1 in targeting the integration.

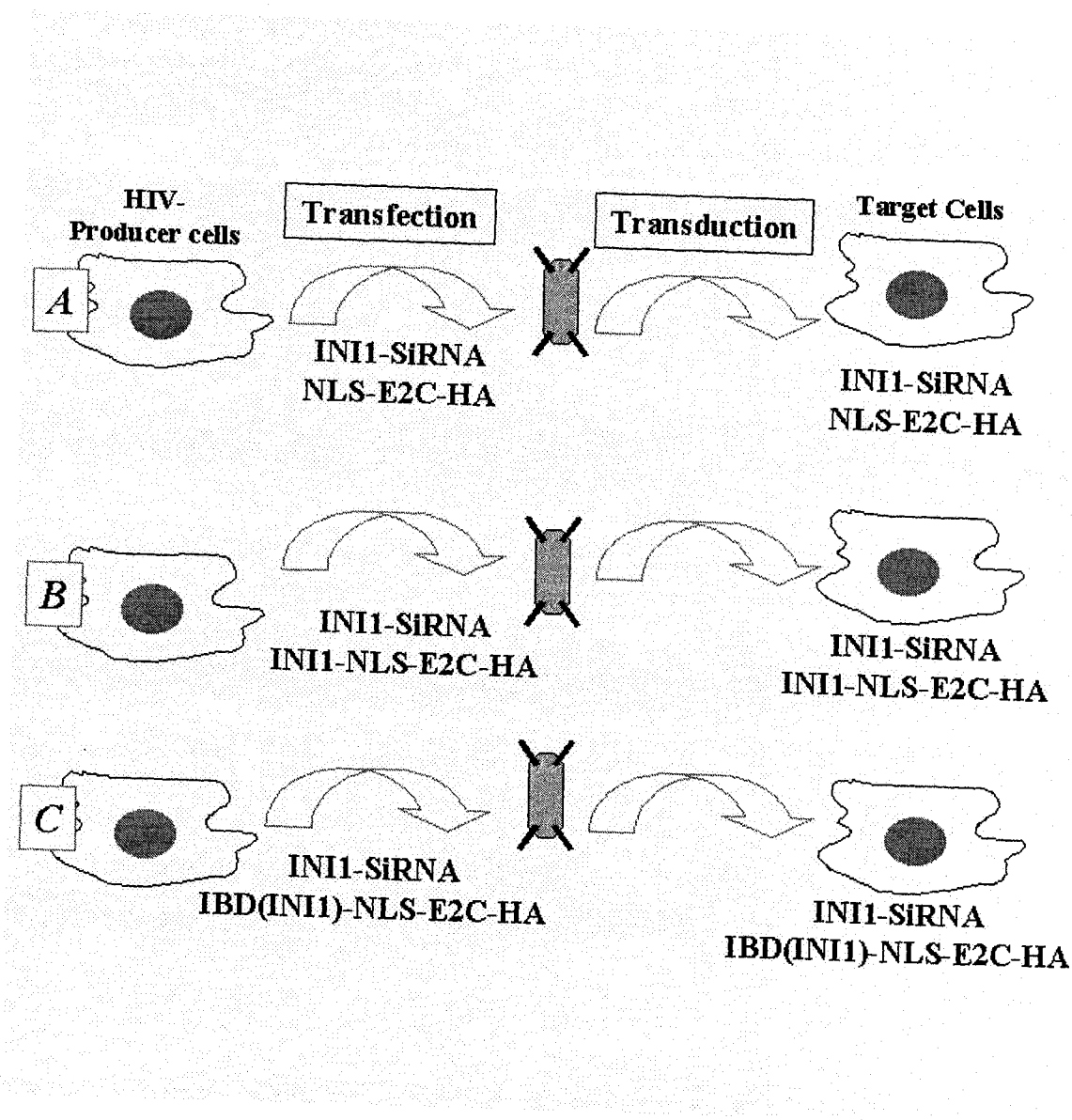


Figure 6. Schematic representation of the *in vivo* retargeting system.

If INI1 is normally involved in integration "targeting", *in vivo* integrations in the presence of INI1-E2C (and in absence of endogenous INI1), should produce a significantly lower enrichment (compared to IBD (INI1)-E2C) of integration at *ErB-b2* because the functional full-length INI1 will compete with the E2C moiety of the chimeric protein to direct the integration to other sites. A similar strategy would apply to LEDGF.

A mammalian expression plasmid based on pSG5 was constructed (**Fig. 7A**) to express a fusion products consisting of a nuclear localization signal (NLS), the zinc-finger protein E2C, and an N-terminal hemaglutinin (HA) epitope tag (pSG5-E2C). The full-length cDNA sequence (without stop codon) of INI1 and LEFGF were added in-frame at the 5'end of the NLS signal in pSG5-E2C to yield the mammalian expression plasmids pSG5-INI1-E2C and pSG5-LEDGF-E2C. The minimal integrase binding domain (IBD) on INI1 (residues 183-294) (318) and LEDGF (residues 340-417) (319) were similarly added to pSG5-E2C to create the mammalian expression plasmids pSG5-INI1(IBD)-E2C and pSG5-LEDGF(IBD)-E2C. Finally, given that our system requires the downregulation of endogenous INI1 and/or LEDGF to be fully effective, we constructed plasmids driving the expression of siRNAs (small interfering RNAs) from a human U6 promoter. In order to down-regulate endogenous INI1 and LEDGF without affecting over-expressed E2C-fused INI1 and LEDGF, the 5'UTR-exon1 and exon-3'UTR junctions were chosen as targets for siRNAs. Three different siRNA constructs for INI1 and LEDGF (**Fig. 7B**) were designed because untranslated regions (UTRs) are not normally fully amenable to siRNA-mediated decay.

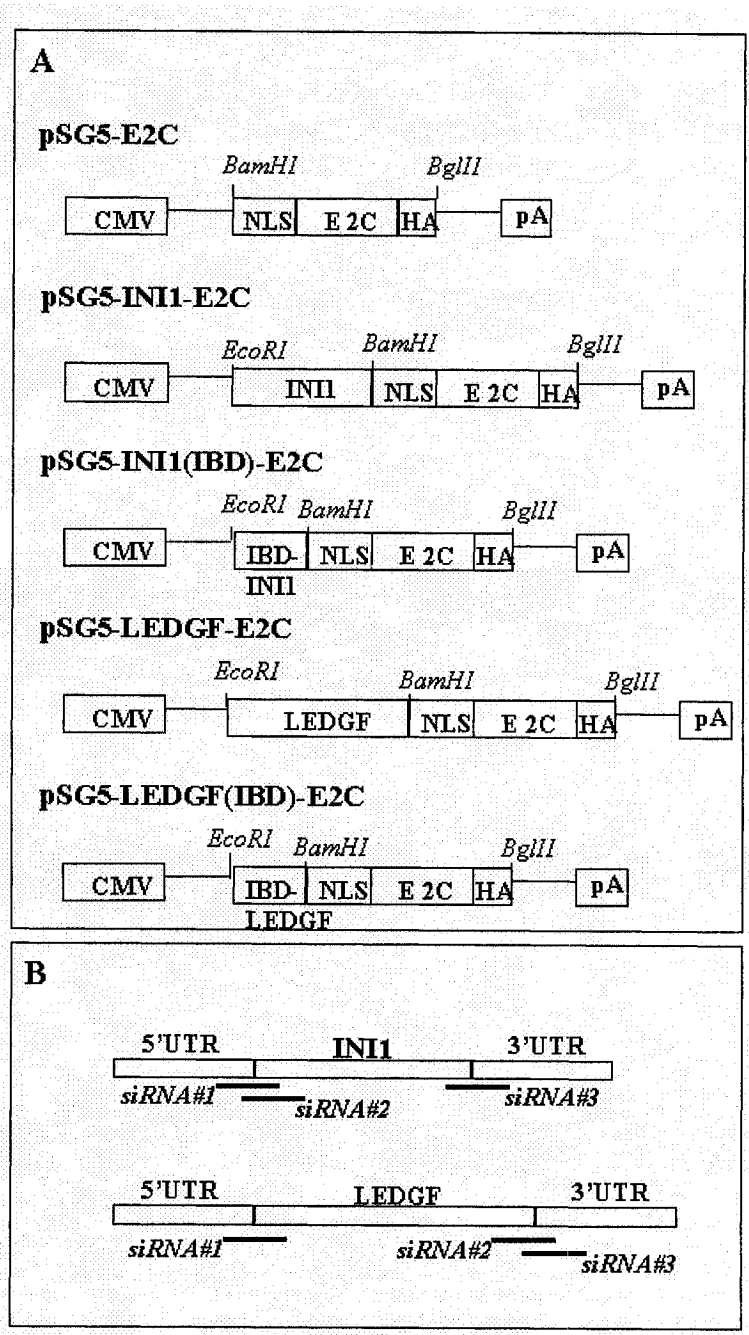


Figure 7. Mammalian expression plasmids constructed for the in vivo model. E2C-fusion constructs (**A**) and siRNA targets (**B**) are depicted schematically.

CONSTRUCTION OF 293GR-LUC PACKAGING CELLS

To facilitate transfection of the INI1-E2C and LEDGF-E2C expression cassettes in conjunction with siRNA expression plasmids, the Rev packaging plasmid (tetracycline-regulated) as well as the Luciferase vector (Rev-dependent) was stably transfected into 293G cells. 293G cells are derived from 293 cells and express the VSV-G envelope glycoprotein under tetracycline repression (312). Linearized pT2Hyg-Rev and pWPT-Luc were co-transfected using a polycationic lipid formulation into 293G cells and transfected cells were selected with hygromycin. Surviving cells were then expanded into clones by limited dilutions in 96-well plates. Twenty-four clones were initially selected for analysis of luciferase expression (**Fig 8**). Of these, height clones (#6, #7, #8, #16, #19, #20, #22, and #24) displaying very high levels of luciferase activity (greater than 150 Relative light units per microgram of protein) were further expanded. Clone #10 was lost due to bacterial contamination. Rev basal and induced expressions were analyzed by semi-quantitative RT-PCR after 5 days of induction (**Fig. 9A**). It was found that clone #6 displayed the highest ratio (induced/basal) of induction, a 2.3-fold increase in expression (**Fig 9B**), and was thus selected to constitute the lentiviral packaging cell line (293GR-Luc).

CONCEPTION OF THE *IN VITRO* INTEGRATION SYSTEM

The existence of an integration "targeting" pathway *in vivo* might have several potential effects on the integration process itself: wider distribution of target sites, enrichment of integration at highly expressed genes, increased rate of integration mediated by the DNA-tethering capability of associated cellular cofactors, and enhanced access to genomic

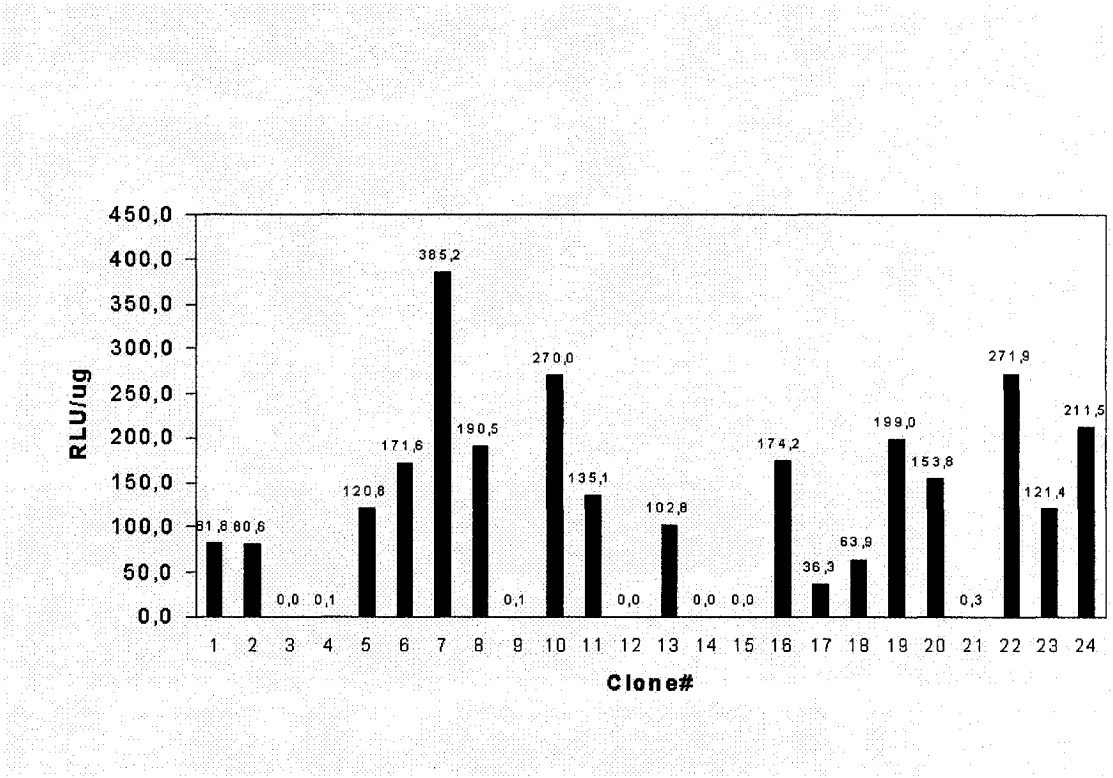
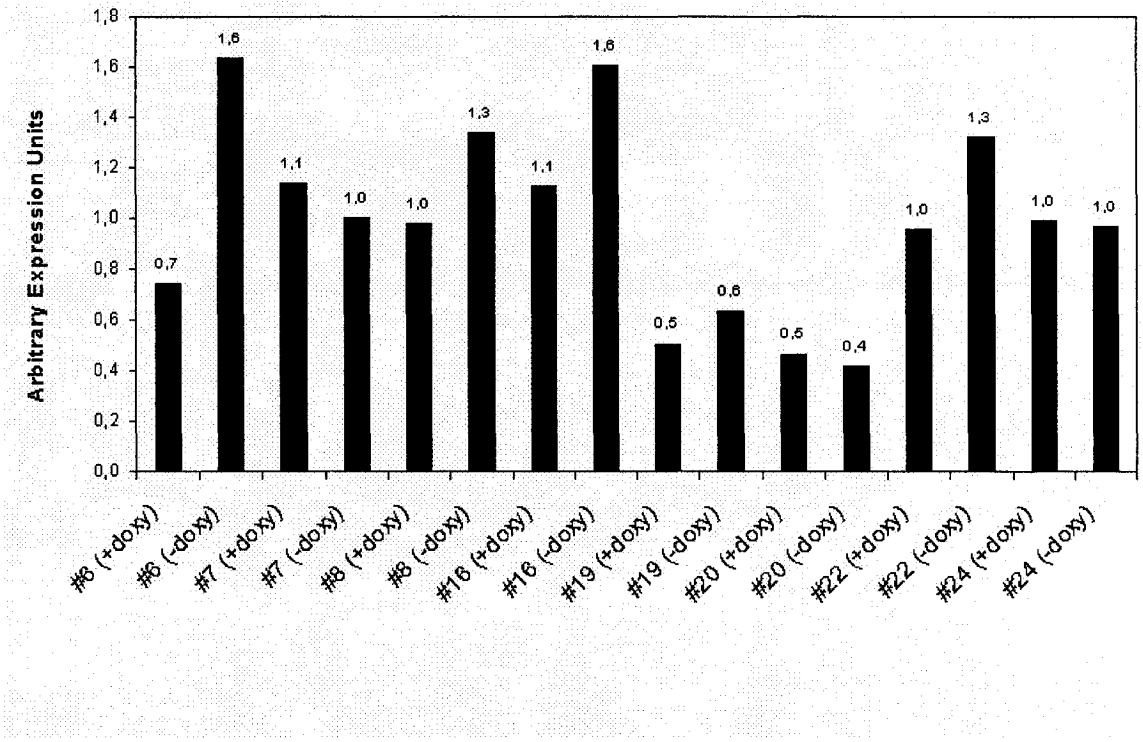


Figure 8. Analysis of the luciferase activity of 293GR-Luc clones.

Two hundred thousand 293GR-Luc clonal cells were platted into the well of a six-well plate. Forty-eight hours later, cells were harvested and assayed for their luciferase activity with a luminometer. Relative light units (RLU) were standardized with the amount of proteins in each reaction.

A



B

293GR-Luc clones	Induction
Clone#6	**2.3**
Clone#7	**0.9**
Clone#8	**1.3**
Clone#16	**1.5**
Clone#19	**1.2**
Clone#20	**0.8**
Clone#22	**1.3**
Clone#24	**1.0**

Figure 9. Analysis of Rev expression in 293GR-Luc clones.

Five hundred thousand cells from selected 293GR-Luc clones were platted with and without doxycycline. Cells were grown for 5 days and RNA was extracted. The level of Rev expression was determined by semi-quantitative RT-PCR. Rev expression data were standardized using human histone 3.3 as a control (**A**). The level of Rev induction was calculated using computer-assisted densitometry (**B**).

DNA through remodeling of chromatin (reviewed in (78;88;295;320). To study, under fixed predetermined parameters, the individual contributions of INI1 and LEDGF to integration targeting, we devised a novel *in vitro* concerted integration model that uses a dynamic synthetic chromatin template to mimic gene transcription (**Fig. 10**). In addition, *in vitro* integration experiments performed with this template should constitute a better model of the *in vivo* mechanisms for HIV integration in comparison to experiments using naked DNA templates or salt-compacted chromatin templates (321). Our system thus relies on the use of purified SWI/SNF core chromatin-remodeling complex to achieve ATP-dependent active chromatin remodeling in our integration assays (322). Purified core histones will be assembled on a plasmid containing nucleosome binding sites with *Drosophila* S190 chromatin assembly extract. *In vitro* chromatin templates generated by this procedure yield nucleosomal arrays of physiological distance and compaction, and have been extensively used to study gene transcription and chromatin remodeling (323;324). Given that INI1 and LEDGF would potentially alter chromatin conformations, this system requires the generation of integrase mutants that are interaction-defective for INI1 and LEDGF. In this manner, the chromatin state of the target DNA will remain constant in the candidate and control experiments since protein content will be identical. The only difference in experimental conditions will reside in the disrupted interaction between the integrase and the selected candidate protein.

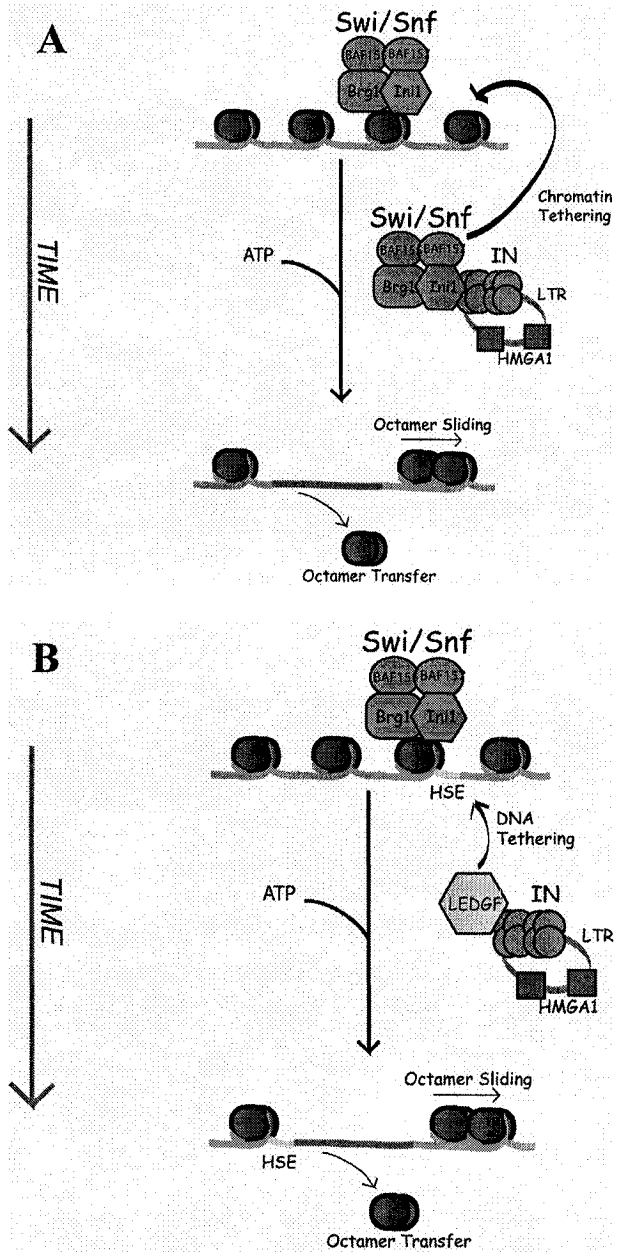


Figure 10. ***In vitro*** **model of integration targeting on chromatin-remodeling template.** At the start of the reaction, the chromatin is fully compacted and integrase is unable to perform integration of the provirus-like substrate. As the reaction progresses, SWI/SNF remodels the chromatin, permitting the efficient tethering of integrase to target sites through INI1 (**A**) and LEDGF (**B**) interactions.

An increased rate of integration and/or altered distribution of integration sites (wild type integrase in comparison to interaction-defective integrase) will demonstrate the active involvement of INI1 or LEDGF in targeting the integration to specific DNA sequences or non-specifically to open chromatin. This could be accomplished by different mechanisms: 1) opening chromatin at the target site to facilitate integration; 2) active tethering of IN to remodeled chromatin; and 3) increase in the affinity of IN with the target DNA (specifically through DNA-tethering capability). If we do not see any involvement of the mechanisms suggested above but still observe increased rate/distribution of integration, it would suggest that INI1 or LEDGF increases the rate of integration through directly stabilizing the integration complex or through enhancing the activity of this complex. If we do not observe any differences in rates or distributions of integration, it would suggest that INI1 or LEDGF perform functions in the viral life cycle other than in integration.

VALIDATION OF THE YEAST TWO-HYBRID SYSTEM FOR INI-IN, LEDGF-IN, AND IN-IN INTERACTION

As mentioned above, the main limitation of the proposed *in vitro* integration model is the requirement for integrase mutants that are defective for interaction with INI1 and LEDGF, while remaining catalytically active *in vitro*. Our primary tool to generate these mutants was the yeast two-hybrid system. Wild type integrase fused to the DNA-binding domain of GAL4 (DBD, Myc epitope-tagged) or the activation domain (AD, HA epitope-tagged) were assayed for their ability to interact with, respectively, AD-INI1 and DBD-INI1 (**Table 6**) in the yeast two-hybrid system. Only the AD-INI1/DBD-IN interaction showed sufficiently low non-specific activity (when assayed with DBD alone or DBD-

Table 6. Specificity of the IN-INI1, IN-LEDGF, and IN-IN interactions in the yeast two-hybrid system.

AD-INI1, AD-LEDGF, and AD-IN were transformed in the AH109 yeast strain and the resulting strains were subsequently transformed with plasmids expressing DBD, DBD-Lamin C, and DBD-IN. Cells were plated in the presence of X-alpha-gal on synthetic complete medium with nutritional restrictions. Number and growth rate of colonies were monitored in conjunction with alpha-galactosidase activity. ++++, deep blue colour, very high numbers of colonies on histidine-deficient medium, growth on histine- and adenine-deficient medium; +++, blue colour, high number of colonies on histidine-deficient medium; ++, light blue colour, high number of colonies on histidine-deficient medium, moderate growth rate; +, light blue colour, low number of colonies, slow growth rate; -, white colour, no or very few colonies.

Fusion proteins	AD-INI1	AD-LEDGF	AD-IN
DBD	++	-	-
DBD-Lamin C	+	-	-
DBD-IN	+++	+++	++++

lamin) to permit further analyses of interaction. In the case of DBD-INI1, the basal activity (when assayed with AD alone) was almost equivalent to the activity with AD-IN, and thus could not be used for interaction analysis (data not shown). The other candidate cellular protein, LEDGF, when fused to AD, demonstrated a strong and specific interaction with integrase, and no background activity when assayed with DBD-lamin or DBD alone. We also sought to determine the structural integrity of our integrase mutants by analyzing their ability to heterodimerize with wild type integrase in the yeast two-hybrid system (**Table 6).** We observed a very specific AD-IN/DBD-IN interaction with minimal background activity (when assayed with DBD-Lamin or DBD alone).

YEAST TWO-HYBRID ANALYSIS OF RESIDUES 47 TO 60.

Given that the INI1 binding region on integrase had previously seemingly been mapped to residues 47-60, we proceeded to analyze the amino acids essential for the INI1-integrase interaction using the yeast two-hybrid system. Every amino acid from position 47 to 60 of integrase was mutated by site-directed mutagenesis, converting charged side chains to hydrophobic residues, and *vice versa.* Integrase mutants were then assayed for their binding affinity with INI1 in the yeast two-hybrid system (**Table 7**). Only M50E seemingly showed a significant reduction but not abrogation of interaction with INI1 in the yeast two-hybrid system. A49D and Q53L also had a slight reduction of interaction with INI1. The other mutations did not affect the binding affinity of integrase with INI1. Surprisingly, the C40S-C43S mutations, which were previously shown to block the binding of INI1, did not show any abrogation of interaction but rather produced an increase in the affinity of IN for INI1. Finally, it is noteworthy that all of the mutants

Table 7. IN-INI1 and IN-IN interactions of integrase mutants at amino acids 47 to 60.

Yeast strains expressing AD-INI1 and AD-IN were transformed with a plasmid expressing a DBD-fused integrase mutant. Cells were plated in the presence of X-alpha-gal on synthetic complete medium with nutritional restrictions. Number and growth rate of colonies were monitored in conjunction with alpha-galactosidase activity. ++++, deep blue colour, very high numbers of colonies on histidine-deficient medium, growth on histine- and adenine-deficient medium; +++, blue colour, high number of colonies on histidine-deficient medium; ++, light blue colour, high number of colonies on histidine-deficient medium, moderate growth rate; +, light blue colour, low number of colonies, slow growth rate; -, white colour, no or very few colonies.

Integrase mutants (DBD)	AD-INI1	AD-IN
IN (wild type)	+++	+++
IN (C40S, C43S)	++++	-
IN (G47D)	+++	+++
IN (E48A)	+++	++++
IN (A49D)	++/+++	++++
IN (M50E)	++	++++
IN (H51F)	+++	++++
IN (G52D)	+++	++++
IN (Q53L)	++/+++	++++
IN (V54D)	+++	++++
IN (D55L)	+++	++++
IN (C56K)	+++	++++
IN (S57V)	++/+++	++++
IN (P58H)	+++	+++
IN (G59D)	+++	+++
IN (I60E)	+++	+++

except G47D, P58H, G59D, and I60E were able to dimerize as efficiently as wild type integrase.

GST PULL-DOWN ANALYSIS OF SELECTED INTEGRASE MUTANTS

GST pull-downs were performed to confirm the interaction results obtained from the yeast two-hybrid system. GST (29 kDa) and GST-INI1 (~ 75 kDa) were first expressed and purified from bacteria (**Fig. 11**). Increasing amounts of GST-INI1 as well a GST were then incubated with *in vitro*-translated wild type integrase to demonstrate the specificity of the binding conditions (**Fig. 12A**). Afterward, selected integrase mutants were *in vitro* translated from the same plasmid used in the two-hybrid system (pDBD-IN, Myc epitope-tagged) and assayed in pull-downs with GST and GTS-INI1 (**Fig. 12B**). However, in contrast to the results obtained in the yeast two-hybrid system, the M50E mutants did not differ from the wild type in regard to its binding affinity with INI1. Likewise, the other mutants assayed (G47D, A49D, and Q53L) demonstrated interaction levels comparable to that of the wild type integrase. When integrase was expressed without epitope tags from pSG5-IN, M50E did not differ in its ability to bind to INI1, as determined by computer-assisted densitometry (**Fig. 12C**). This conservation of interaction even in the absence of the Myc tag, a possible interfering factor, suggested that the residues 47, 49, 50, and 53 were not involved in the INI1 interaction. Moreover, these results also implied that the INI1-binding domain on the integrase might not be localized in the 47-60 region.

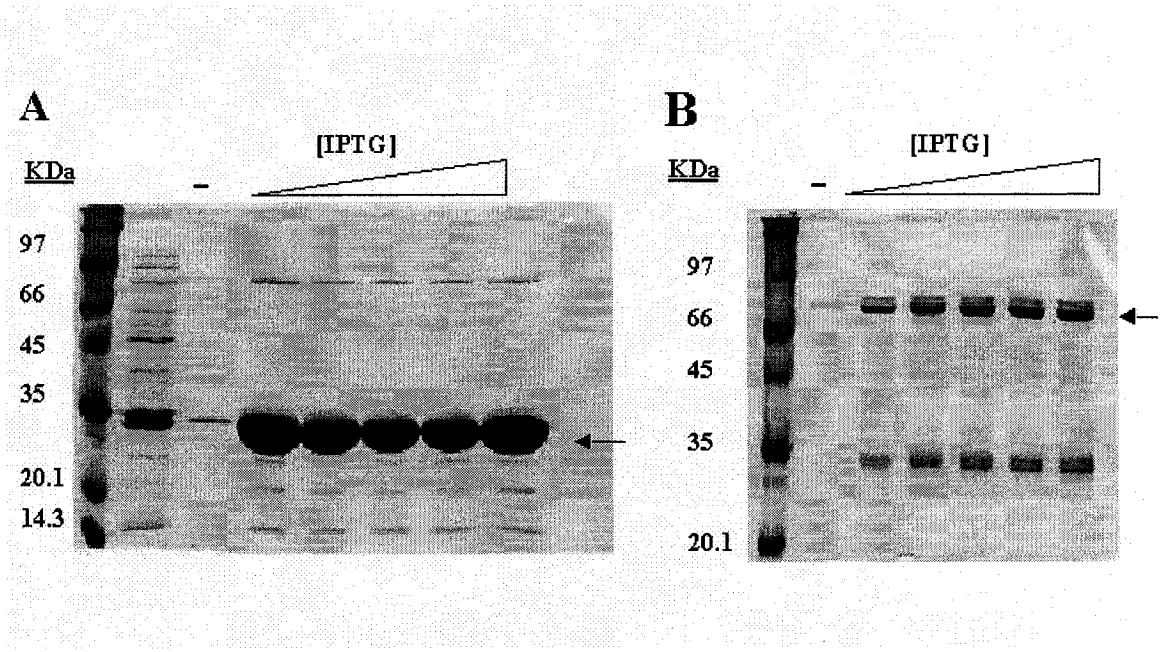


Figure 11. Expression and purification of GST and GST-INI1.

GST (29 kDa) (**A**) and GST-INI1 (75 kDa) (**B**) were expressed in E. coli (BL21) over a range of IPTG concentrations, were purified on glutathione-sepharose beads, and resolved on a 10% denaturing polyacrylamide gel. Arrows show the bands corresponding to the purified proteins.

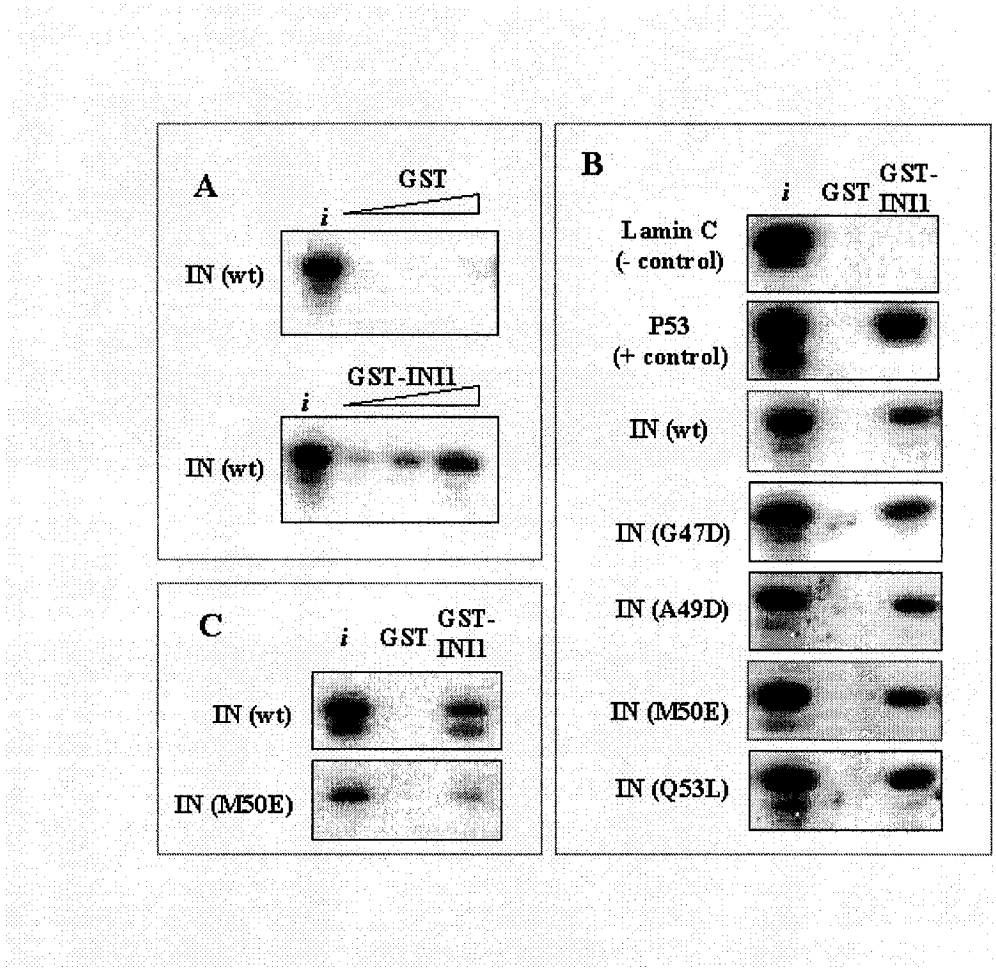


Figure 12. GST pull-down analyses of the interaction between INI1 and selected integrase mutants.

Increasing amounts of GST or GST-INI1 bound to glutathione-sepharose beads were incubated with 10 ul of ^{35}S-labelled *in vitro*-translated (using rabbit reticulocyte lysate) wild type integrase (IN) to demonstrate the specificity of the interaction in our binding conditions. Beads-bound proteins were denatured and resolved on a 10% SDS-PAGE (**Panel A**). Selected IN mutants (Myc-tagged), ^{35}S-labelled and *in vitro*-translated, were assayed for their binding affinity with GST-INI1 (**Panel B**). Wild type and M50E integrase (native), ^{35}S-labelled and *in vitro*-translated from pSG5-IN and pSG5-IN (M50E), were assayed for their binding affinity with GST-INI1 (**Panel C**). ***i*** : input, 10% of the ^{35}S-labelled *in vitro*-translated protein used in the binding assay.

INVESTIGATION OF OTHER POTENTIAL INI1-BINDING DOMAINS ON INTEGRASE

In order to identify the INI1-binding domain on integrase, we generated a series of integrase deletions at key positions, expressed in fusion with the DBD of GAL4. Unexpectedly, all the truncated integrase mutants were still fully capable of interacting with INI1 **(Table 8)**. The integrase truncated mutants that comprised residues 1-47 and 1-54 showed a level of interaction similar to the non-specific activity of the DBD alone. All of the N-terminal truncated integrase were as proficient as the wild type to bind to INI1. Moreover, two of the deletion mutants (55-288 and 61-288) assayed were later found to contain frame shift mutations at the junction of the Myc epitope tag and integrase, implying that all of the observed interactions between integrase and INI1 in the two-hybrid system were non-specific.

Table 8. Interactions of INI1 and truncated integrase mutants in the yeast two-hybrid system.

Yeast strain expressing AD-INI1 was transformed with a plasmid expressing a DBD-fused integrase deletion mutant. Cells were plated in the presence of X-alpha-gal on synthetic complete medium with nutritional restrictions. Number and growth rate of colonies were monitored in conjunction with alpha-galactosidase activity. ++++, deep blue colour, very high numbers of colonies on histidine-deficient medium, growth on histine- and adenine-deficient medium; +++, blue colour, high number of colonies on histidine-deficient medium; ++, light blue colour, high number of colonies on histidine-deficient medium, moderate growth rate; +, light blue colour, low number of colonies, slow growth rate; -, white colour, no or very few colonies.

Integrase deletions	**AD-INI1**
IN (wild type)	+++
IN (1-47)	++/+++
IN (1-54)	++/+++
IN (38-288)	+++
IN (44-288)	+++
IN (48-288)	+++
IN (55-288)*fs**	+++
IN (61-288)*fs**	+++

* Frame-shift mutations

DISCUSSION

One of the least understood aspects of the HIV life cycle is the series of events beginning with nuclear import of the pre-integration complex and leading to the integration of the proviral DNA inside of the host genome. The chemical mechanisms of the integration reaction have been known for over a decade, but the molecular pathways underlying the nuclear trafficking of the pre-integration complex as well as the selection of integration site have remained elusive. Uncovering these mechanisms could lead to major developments in the fields of gene therapy and anti-retroviral drug design.

The recent development of acute lymphoproliferative disorders in three children who were apparently "cured" from severe combined immunodeficiency by retroviral transduction of hematopoietic cells (273) highlights our deficient understanding of the fundamental aspects of retroviral integration. The risk of vector insertional mutagenesis in gene therapy protocols was long thought to be negligible. However, retroviruses have evolved with the principles of maximizing proviral expression and viral particle production (175;183;325) . A mechanism to target the integration to favorable sites for expression thus appears to be a fundamental component of retroviruses and associated vectors (96). Identification of the cellular proteins involved in integration targeting could lead to the development of a new class of retroviral gene transfer vectors that would integrate in predetermined, "safer" loci of the genome. Such vectors would greatly improve the safety of gene therapy since the risk of malignancy induced by insertional mutagenesis would be greatly reduced or eliminated. Moreover, identification of these cellular proteins and of their domains of interaction with viral proteins may permit the

development of novel anti-HIV therapies directed at these cellular proteins. These targets should be less prone to the emergence of drug resistance phenotypes because of the reduced rates of mutation of cellular DNA polymerases compared to the viral RT. In these perspectives, we have designed two novel HIV integration models to investigate the potential roles of the cellular transcription factors INI1 and LEDGF in targeting the proviral integration to highly expressed genes.

IN VIVO MODEL OF INTEGRATION TARGETING

Other investigators have attempted to study integration targeting cellular cofactors (mostly INI1 and LEDGF) through the use of siRNA or cofactor-deficient cancer cell lines (46;71;295;296). However, in most cases, there were no noticeable effects on integration frequency, presumably because of redundancies in the targeting pathway. Such redundancies would ensure that integration does not occur in unfavorable regions of the genome (88). Gene deserts, centromeric heterochromatin, and very highly expressed cellular genes have been shown to have a major detrimental impact on proviral expression and ultimately virus particle production (97;326). High levels of viral titer appears to be important for the early stages of infection and consequently for the development of AIDS (183;325).

Therefore, the study of HIV integration targeting requires an *in vivo* system in which the involvement of each cellular cofactor can be studied, irrespective of the redundant function of other proteins. In this perspective we designed a novel system that redirects the integration to a specific genomic locus when the studied cofactor is actually involved in integration targeting. The cellular candidate proteins INI1 and LEDGF would be expressed in fusion with the synthetic zinc finger protein E2C, which bind with high

affinity and specificity to a unique 18-bp sequence in the human *ErbB-2* gene. As mentioned above (**Fig. 6**), in vivo integration performed with such constructs should lead to a competition of integration target sites if the studied cofactor is involved in the targeting pathway. The DNA-binding domain of the cellular cofactor should compete with the E2C moiety to target the integration to their respective DNA cognate sequences. Thus, differential degrees of enrichment of integration in *ErbB-2* when the infection is performed in the presence of the E2C-fused full-length cofactor or the E2C-fused minimal integrase binding domain (IBD), would determine whether a candidate protein is involved in the integration-targeting pathway.

Here, we report the initiation of the proposed integration retargeting system. We generated the plasmid expressing INI1 (full length), INI1 (IBD), LEDGF (full length), and LEDGF (IBD) fused to E2C (**Fig. 7A**). Integration retargeting with the E2C fusion constructs would be more effective in the absence of endogenous IN11 or LEDGF. In this perspective, we constructed siRNA expression plasmids to down-regulate endogenous INI1 and LEDGF. These siRNAs target the junction of 5'UTR and Exon1 or the junction of the last exon with 3'UTR (**Fig. 7B**). That way, endogenous INI1 and LEDGF will be down-regulated without affecting the over-expressed E2C-fused constructs. In order to facilitate the transient transfection of multiple plasmids encoding siRNAs and E2C-fusion derivatives, we also constructed a stable inducible lentiviral packaging cell line. The cell line was built from 293G cells, which express the VSV-G envelope glycoprotein under tetracycline repression (312). The Rev packaging plasmid (tetracycline-regulated) as well as the luciferase vector (Rev-dependent) was stably transfected into 293G cells. Individual clones were expanded and characterized for their

levels of luciferase activity as well as Rev inducible expression (**Fig. 8 and 9**). The clone that displayed the best expression profile was selected to constitute the 293GR-Luc cell line. To avoid pleiotropic defects on viral particle production and infectivity due to the absence of INI1 or LEDGF, we deleted the integrase from the Gag-Pol expression plasmid and expressed it as a Vpr-fusion product (314;315). This strategy was previously shown to alleviate the drastic reduction of particle production as a result of INI1 abrogation (296). Finally, both Gag-Pol and integrase will be supplied by transient transfection in induced (after removal of doxycyline) 293GR-Luc cells to produce HIV-based vectors. The capability of the constructed 293GR-Luc cells to support high viral titer production still remains to be determined. Because these types of stable packaging cell line do not easily yield high titer of "gutted" HIV-based vectors (313), we also explored an alternate strategy to produce HIV virions using a transiently transfected one-cycle infection construct. To this aim, the integrase was deleted from a fully infectious clone of HIV and was expressed in *trans* as a Vpr-fusion product (73). We also deleted the HIV envelop from the viral genome and replaced it with the trans-complementation of the VSV-G glycoprotein, in order to increase the biosafety level of our constructs. Whichever of the two viral production strategies provide a high viral titer with a significant expression of siRNA and E2C-fusion products would constitute the packaging cell line for our future investigation. The next and final steps in the development of this experimental system consist of testing the effectiveness of the designed siRNAs and determining the ability of E2C-fusion constructs to interact with the integrase. Finally, the pairing of this model with a high throughput method for determination of integration sites would be a major asset. Several strategies based on large-scale, automated LM-PCR (89) or on genomic FISH (fluorescence *in situ* hybridization) (91) could be implemented.

IN VITRO MODEL OF INTEGRATION ON CHROMATIN TEMPLATE

To study the potential involvement of individual cellular protein in the integration-targeting pathway, we devised a more complex model of *in vitro* integration. This system would be based on a nucleosome-coated template, actively and gradually remodeled by the ATPase activity of the core units of the SWI/SNF chromatin-remodeling complex. These types of *in vitro* chromatin-remodeling templates have been widely used in the study of chromatin remodeling as well as transcription regulation and have been instrumental for our current understanding of these mechanisms (323). The use of these templates could thus have major implications in the elucidation of some of the least understood aspects of retroviral integration. Indeed, a recent study demonstrated that the HIV integrase was unable to perform integration on stable salt-compacted chromatin templates, whereas the ASV integrase was fully active (321).

In our proposed *in vitro* system, at the start of the integration reaction, the target template would be inaccessible to the integrase because of the full histone compaction. However, as the reaction proceeds in time, the activity of the SWI/SNF complex would gradually open the chromatin, allowing the integrase to access the target DNA and to perform integration of the provirus-like substrate. The positive role of cellular proteins, such as INI1 and LEDGF, could result in an increased rate of integration (preferential access to the opening chromatin) or wider distribution of integration sites (active remodeling of chromatin at integration site) (**Fig. 10**). The main limitation of the proposed model is that INI1 or LEDGF, when added to the reaction, would by themselves modify the structure and compaction of the chromatin, and would thus influence the integration reaction without the need for a functional interaction between the integrase and the cofactor.

Keeping the chromatin condition constant between different experimental settings therefore requires the generation of integrase mutants unable to interact with INI1 and/or LEDGF, but sill catalytically active *in vitro*. That way, the only variable parameter would be the interaction between integrase and its potential cofactors INI1 and LEDGF.

The interaction between HIV integrase and INI1 was uncovered more than a decade ago by S. Goff and colleagues with the help of the yeast two-hybrid system (297). In the same report, the authors showed that amino acids 47 to 60 of the integrase was likely responsible for this interaction (297). We thus focused on this region of integrase using the yeast two-hybrid system as a primary tool in order to generate integrase mutants defective for INI1 interaction. In our yeast two-hybrid system, variable degrees of non-specific activity were observed in the presence of INI1. However, this background noise was initially deemed unobtrusive since it was lower than the activity of the positive interaction between INI1 and integrase. We then proceeded to mutate by site-directed mutagenesis every residue of the 47-60 region and studied in the yeast two-hybrid system the resulting effects on the interaction between INI1 and integrase as well as on integrase dimerization. We found that only the M50E mutation reduced the level of activity down to the background noise (**Table 7**). Surprisingly however, we did not observe an abrogation of interaction with the C40S-C43S mutations, in contrast to previously published observations (297). In our case, the C40S-C43S mutations were found to increase the affinity of the integrase for INI1. In addition, we showed that none of the studied mutations significantly destabilized the structure of the integrase, as demonstrated by the ability of all integrase mutants to effectively dimerize (**Table 7**). We then sought to confirm with GST pulldown experiments that the M50E mutation abolished the

interaction with INI1 (**Fig. 12**). However, we did not observe any reduction of interaction between the M50E mutant and INI1, suggesting that the apparent reduction of interaction, obtained in the two-hybrid system, was only an artefact. In addition, these GST pull-down results showed that the 47-60 region of the integrase probably did not mediate the interaction with INI1, suggesting the necessity to identify the actual domain of interaction. To this aim, we designed a set of specific N-terminal and C-terminal integrase deletions in order to obtain a general idea about the location of the interaction domain. However, when assayed in the two-hybrid system, none of these deletions mutants produced a significant reduction of interaction (**Table 8**). Moreover, two of the truncated mutants assayed (55-288 and 61-288) were later found to contain a frame shift mutation at the junction between the myc epitope tag and the N-terminus of the truncated integrase. These last surprising results demonstrated that all of the observed interactions between integrase and INI1 in the yeast two-hybrid system were the result of the overlooked background activity of INI1. Because of that, it is impossible to conclude whether the 47-60 region of integrase is effectively mediating the interaction with INI1.

The variable degree of background activity in the yeast two-hybrid system (**Table 6, 7, and 8**) appeared to be specific for INI1, since LEDGF and integrase (**Table 6**) did not induce any non-specific activation of the reporter genes. Several non-exclusive factors may explain this highly variable non-specific activity of INI1. One possible explanation is that INI1 might be able to interact directly with the DBD of GAL4. However, the original report on the characterization of INI1 argues against this idea. Indeed, Kalpana et al. showed that there were no cross-interaction between AD-INI1 and DBD in a GAL4-based yeast two-hybrid system (297). Another possibility is that INI1 might interact with

the c-myc epitope tag in the linker region between DBD and the fusion protein. INI1 was previously shown to interact with the proto-oncogene c-myc in its basic helix-loop-helix and leucine zipper domains (bHLH-Zip) (298), which include the sequence of the myc epitope tag. Nevertheless, the apparent lack of interaction between Myc-tagged Lamin C and GST-INI1 in the pull-down assays challenges this possibility. Moreover, the frame-shifted truncated integrase mutant 61-288 (table 8) had a recombination in the three last amino acids of the myc tag and was still fully competent at activating the transgenes. This would mean that INI1 could interact with the linker region from the DBD up to the T7 promoter, since this sequence was not present in the GST pull-downs.

To resolve this problem, we constructed a pGBKT7-derived plasmid, in which the linker and c-myc epitope tag were deleted. When this new plasmid was assayed in the two-hybrid system for possible interaction with AD-INI1, both DBD and DBD-IN were unable to activate transgene transcription (data not shown). This is surprising because INI1 is known to interact with integrase in yeast (297). In contrast, AD-LEDGF was able to interact with DBD-IN (without linker) as strongly as with Myc-tagged DBD-IN (data not shown). It should be noted that integrase was previously shown to be toxic in yeast, and that this lethal adverse effect of integrase was dependent on endogenous SNF5, the yeast homologue of INI1, strengthening the potential role of INI1 in integration (327). Therefore, the combined high expression levels of INI1 and integrase in yeast cells would have been highly toxic, thus selecting INI1-expressing cells with no or very little expression of integrase. In the case of the no-linker plasmid, INI1 non-specific activity was abolished and consequently no transgene activation was observed. Furthermore, when deletions or mutations in the integrase destabilized its structure or blocked the

interaction with INI1, the degree of toxicity would be reduced and the level of INI1-mediated background activity would be increased. This would explain why the C40-C43S mutation did not block INI1 interaction, as previously reported (297), but rather increased it (**Table 7**). A simple experiment to prove this hypothesis would be to perform two-hybrid analysis between INI1 and inactivated integrase (D116A) expressed in fusion with DBD (without linker).

Despite all these difficulties with the study of integrase using the yeast two-hybrid system, we think that it nevertheless represents a valid and effective means to identify interaction-defective mutant integrase. In this context, other investigators recently generated, using the yeast two-hybrid system and a random library of integrase mutations, a mutant integrase (Q168A) defective for interaction with LEDGF, but still catalytically active *in vitro* in one-end strand-transfer reactions (71). Virions harboring integrase (Q168A) were defective for integration but not nuclear import. Over-expressed integrase (Q168A) were also defective for chromatin localization. This study highlights the potential role LEDGF in integration, but particularly in integration targeting (71). Finally, the role of LEDGF in integration site selection could be studied in more details using our proposed *in vitro* model and the Q168A mutant.

In summary, much work remains to be done in order to validate the novel *in vitro* integration model proposed herein. Research efforts should concentrate on generating the chromatin template and performing integration experiments on these templates.

SIGNIFICANCE OF RESULTS

In conclusion, in the aim of studying the role of INI1 and LEDGF in HIV integration targeting mechanisms, we have initiated the construction of two novel models of integration: 1) an *in vivo* integration retargeting system based on the DNA-binding properties on the synthetic protein E2C and 2) an *in vitro* integration assay using actively remodeling chromatin templates. For the *in vivo* system, we have generated a stable inducible lentiviral vector-packaging cell line as well as INI1 and LEDGF E2C-fused mammalian expression plasmids. Moreover, we have constructed siRNA-expression plasmids to knock down endogenous INI1 and LEDGF expression without affecting the E2C-fused constructs. In the context of the *in vitro* assay, we have analyzed the potential role of residues 47 to 60 on the integrase in mediating the interaction with INI1. These interaction studies are needed in order to generate INI1-interaction defective integrase mutants, a primary requirement of our novel *in vitro* integration model. However, we have encountered several problems in the yeast two-hybrid system likely due to INI1-mediated non-specific interactions and integrase toxicity. Therefore, we have been unable to determine whether or not this region of the integrase mediates the interaction with INI1.

The identification and characterization of cellular factors involved in the HIV integration site selection could be of capital importance for the generation of new anti-retroviral therapies. Indeed, drugs targeting cellular proteins rather than viral proteins would likely circumvent the capacity of the virus to generate drug-resistant strains. In that context, the two novel integration models proposed herein could contribute significantly to the elucidation of the integration-targeting pathway. Moreover, the yeast two hybrid and

GST pull-down assays discussed in this report could serve as a platform for the screening of potential drugs blocking the interaction between integrase and cellular proteins such as INI1 or LEDGF. The yeast two-hybrid system was recently successfully used to identify small peptide inhibitors of Vpr-mediated cell cycle arrest, highlighting the potential of such models as drug discovery tools (328). Finally, the elucidation of the targeting pathway could lead to significant advances in retroviral vector designs for gene therapy. The recent adverse cases of leukemia in three SCID-X1 children (269), who had been cured by gene therapy, have considerably diminished the ethical and financial support toward gene therapy (329-331). The development of new classes of vectors that could integrate at specific "safe" loci of the genome would alleviate the problems related to insertional mutagenesis and would redeem gene therapy's reputation (288;332).

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