Cloning and Characterization of MRI, a Modulator of Retroviral Infection

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Abstract

Although significant progress has been made in elucidating the life cycle of HIV and identifying host cell proteins which interact with HIV, a much greater understanding of these processes is necessary in order to eradicate this disease. The studies presented in this dissertation further examine these processes and identifies a new host cell protein, MRI, which appears to be involved in the poorly understood step of viral uncoating.

The first part of my project involved the creation, characterization, and utilization of a novel toxic retroviral vector encoding barnase, a non-specific protease derived from *Bacillus amyloliquefaciens*. In conjunction with an inhibitor of barnase (barstar), I showed that barnase-based retroviral vectors were capable of inducing cell death in a wide variety of cell types and that the presence of barstar was essential for the creation of high titer retroviral vectors.

The second part of my project was to was to create a heavily mutagenized hamster cell line, V79-4, and repeatedly challenge this cell line with the toxic retroviral vector in order to isolate clonal populations of cells resistant to retrovirally mediated transduction. Ultimately, I isolated two cell lines, 31-2 and 67-1, which we characterized further. 31-2 was determined to be least five fold resistant to both MLV- and lenti-viral at a stage in viral replication post reverse transcription. 67-1 was minimally ten fold resistant to both MLV- and lenti-viral infection at a stage between viral entry and uncoating. We demonstrated that the mutation in 67-1 implicated the proteasome and identified a gene we designated MRI that reversed the inhibition of infection in the mutated 67-1 line. The implication of MRI in the retroviral lifecycle identifies a new protein that could facilitate the creation of novel host cell based therapy for HIV.

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CHAPTER 1

Background

Human immunodeficiency virus 1 (HIV-1) is a member of the *Retroviridae* family in the *lentivirus* genus. HIV-1 is the causative agent of acquired immunodeficiency syndrome (AIDS) and according the latest World Health Organization (WHO) statistics through 2007, over 33 million people worldwide are infected with HIV and more than 25 million people have died from AIDS since 1981 (UNAIDS, 2008). Given the devastating social and economic impact of the disease, there has been a concerted effort to understand both the biology of the virus and the disease in order to facilitate antiviral drug development.

HIV-1 Discovery and Disease Statistics

HIV-1 was first discovered in 1983 by Luc Montagnier and his team at the Pasteur Institute in France (Barre-Sinoussi et al., 1983). One year later the results were confirmed by Robert Gallo (Zagury et al., 1984) and Marvin Reitz (Mitsuya et al., 1984). It is generally accepted that Montagnier's group was the first to discover the virus but that Gallo's group made a significant contribution by showing that HIV actually caused AIDS. Since HIV was discovered, two distinct types have been identified that infect humans, HIV-1 and HIV-2 (Clavel et al., 1986). HIV-1, as compared to HIV-2, is more virulent, more easily transmitted, and is the source of the majority of HIV infections throughout the world (McCutchan, 2006). HIV-2 is mainly confined to western Africa (Clavel et al., 1987; McCutchan, 2006). Both species of HIV have origins in nonhuman primates. HIV-1 evolved from the simian immunodeficiency virus (SIV) found in the

chimpanzee subspecies, *Pan troglodytes troglodytes* (SIVcpz) (Gao et al., 1999), and HIV-2 evolved from a distinct SIV strain found in sooty mangabey monkeys from Guinea-Bissau (Hirsch et al., 1989). It is unclear when SIV made the jump from a being primate virus to the current human virus.

Since the discovery of HIV, its infection rate has reached near pandemic proportions. In 2007 alone, nearly three million people became HIV positive and more than two million people died due to AIDS or AIDS related complications, illustrating that high rates of infection and AIDS related death still exist today. Sub-Saharan Africa is home to just over 10% of the world's population, however it is also home to two-thirds of the world's HIV positive individuals including four countries where the percentage of HIV positive individuals is at or above 30% of the population. As a comparison, less then 0.3% of the United States population is HIV positive. Though high rates of HIV infection first occurred in homosexual men and IV drug users, infection has also significantly expanded beyond these groups as 50% of the adults infected with HIV are now female. These statistics are very troubling and in order to reverse the increase in infected individuals, a greater knowledge HIV and its mechanism of infection is needed.

HIV Organization and Lifecycle

Organization of the viral genome and virus

HIV-1 is a retrovirus classified in the *lentivirus* (lenti meaning slow) genus. A retrovirus is an RNA virus that is replicated in a host cell via the enzyme reverse transcriptase to produce DNA from its RNA genome. This DNA is then incorporated into the host's genome by an integrase enzyme. One feature that distinguishes

lentiviruses from other retroviruses, for example the gammaretrovirus Moloney Murine Leukemia Virus (MLV), is that lentiviruses can infect non-dividing cells (Weinberg et al., 1991) whereas other retroviruses are unable to cross the nuclear membrane and therefore require cell division to gain access to the nucleus and integrate (Roe et al., 1993). A second feature that sets HIV apart from gammaretroviruses is the presence of accessory proteins (Nef, Vif, Vpu, and Vpr) and regulatory proteins (Tat and Rev). These accessory and regulatory proteins facilitate HIV replication and cause some of the toxicity associated with wildtype viral infection. The genetic material of HIV is a 9 kb dual copy RNA containing the *Gag, Pol, Env, Nef, Vif, Vpu, Vpr, Tat*, and *Rev* genes flanked by long terminal repeats (LTRs, Fig. 1-1). Proteins coded for by these genes are generated by multiple alternative splicing events from a single mRNA. The *Gag* gene gives rise to a 55 kDa polyprotein that, upon proteolytic processing, generates the matrix (MA [p17]), capsid (CA [p24]), nucleocapsid (NC [p7]), and p6 proteins (Gottlinger et al., 1989). The *Pol* gene gives rise to the reverse transcriptase (RT), protease (Pro), and integrase (Int) (Debouck et al., 1987). The *Env* gene encodes a 160 kDa polyprotein that generates the envelope proteins Gp120 and Gp41 (Robey et al., 1985). With only 15 encoded proteins, HIV possesses all the pieces necessary to make complete virus (Fig. 1-2), however assembly of the pieces requires HIV to behaves as an obligate intracellular parasite, harnessing the host cell machinery to complete its life cycle (Fig. 1-3).

HIV lifecycle: entry

The first step in the HIV life cycle is for the virus to find its target cell. The Gp120 envelope protein of HIV targets CD4 positive T lymphocytes, however peripheral

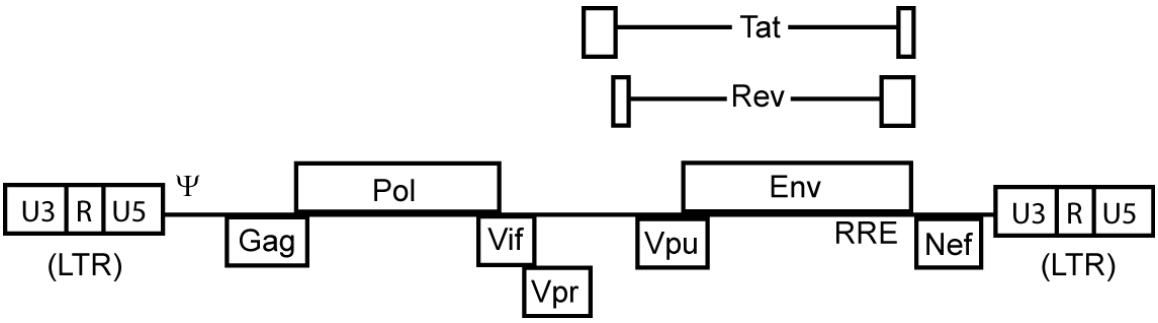
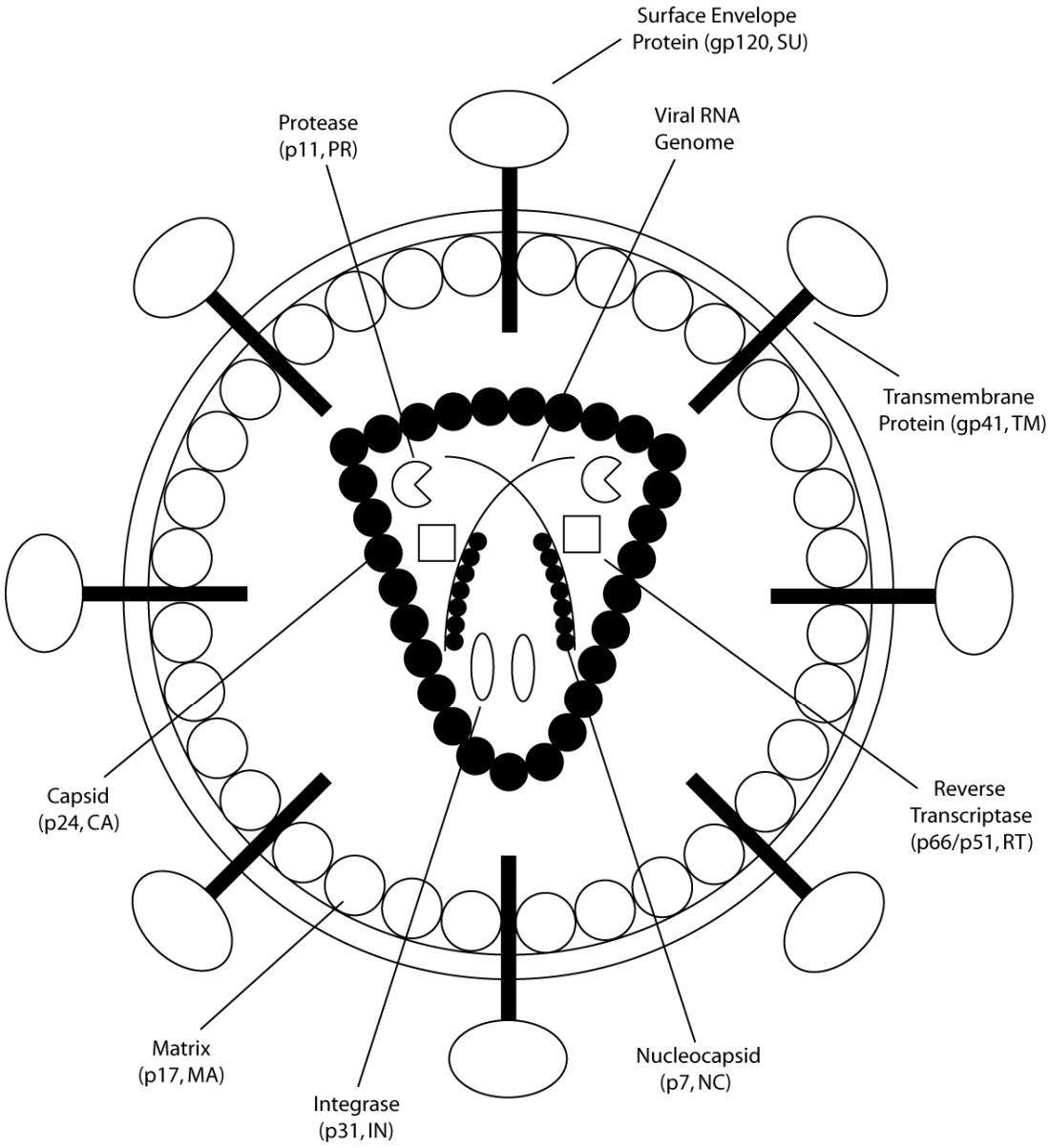

Tat
Rev
U3
R
U5
(LTR)
Ψ
Gag
Pol
Vif
Vpr
Vpu
Env
RRE
Nef
U3
R
U5
(LTR)

Figure 1-1

Organization of the HIV genome.

HIV encodes only fifteen proteins and the genome is flanked by long terminal repeats (LTRs) that regulate gene expression. Gag encodes the structural proteins matrix, capsid, nucleocapsid, and p6. Pol encodes the catalytic proteins protease, reverse transcriptase, and integrase. Env encodes the envelope proteins Gp120 and Gp41. Nef, Rev, Tat, Vif, Vpr, and Vpu are accessory proteins with a variety of functions.



After Freed, 2002

Figure 1-2
Diagram of a mature HIV virus.

Locations of the major proteins encoded by the viral genome within the mature virion are indicated.

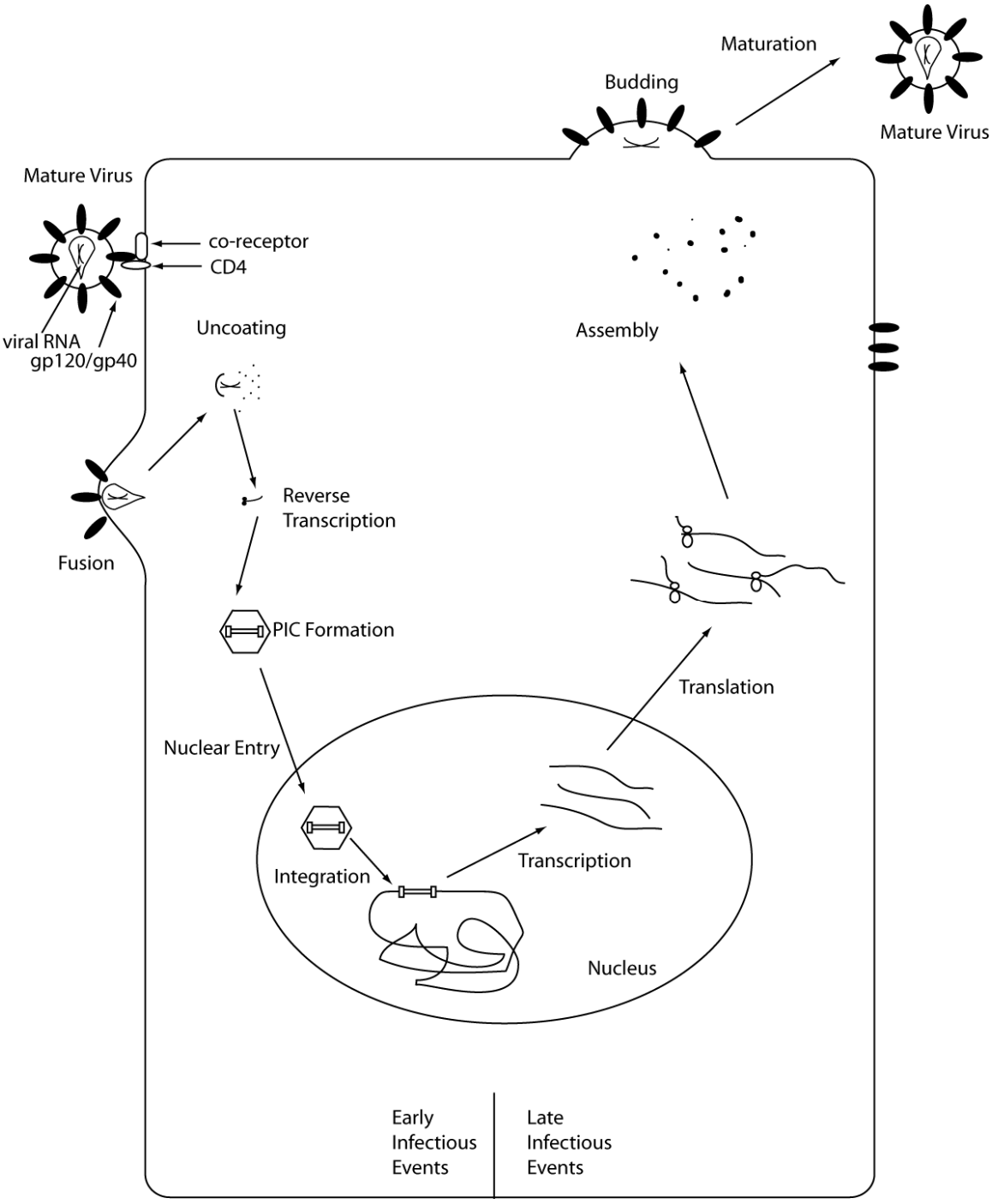

Maturation
Budding
Mature Virus
Mature Virus
co-receptor
CD4
viral RNA
gp120/gp40
Uncoating
Assembly
Reverse
Transcription
Fusion
PIC Formation
Translation
Nuclear Entry
Integration
Transcription
Nucleus
Early
Infectious
Events
Late
Infectious
Events

Figure 1-3

HIV lifecycle.

The HIV lifecycle is very ordered. The mature virus binds to the target cell and there is a fusion event which causes the contents of the virus to gain access to the cytoplasm. In the cytoplasm the virus uncoats, begins reverse transcription, and forms a preintegration complex (PIC). This PIC crosses the nuclear membrane, viral cDNA integrates into the host genome, and transcription of the viral genome is initiated. The viral RNA is transported out to the cytoplasm where it is translated and the proteins are assembled to generate new virus that bud out from the infected cell. A final maturation step occurs post budding, making the virus capable of infecting new targets.

blood mononuclear cells have also been shown to be infectable (Scarlatti et al., 1997). Gp120 binds specifically to CD4 on the target cell and this induces a conformational change in the envelope proteins that gives the virus access to additional chemokine co-receptors on the cell surface (Chan and Kim, 1998; Kwong et al., 1998). HIV interacts with either one of two chemokine co-receptors *in vivo* CXCR4 and CCR5 (Doms and Trono, 2000). Interestingly, up to 13% of individuals of Northern European decent harbor a naturally occurring 32 base pair deletion in the CCR5 receptor which prevents the receptor from ever reaching the cell surface (Liu et al., 1996; Martinson et al., 1997). Those individuals who are homozygous for this mutation are virtually immune to HIV. These data indicate the critical nature of CCR5 in HIV progression and transmission and in 2008 a new anti-retroviral drug specifically targeting CCR5-tropic HIV-1, Maraviroc, garnered full approval by the FDA after a successful clinical trial (Dorr et al., 2005). The binding of Gp120, CD4, and the chemokine co-receptors triggers an additional conformational change within Gp41 (Chan and Kim, 1998). This conformational change triggers a "harpoon" like mechanism that penetrates the target cell membrane and allows fusion between the viral and cell membranes. This fusion event deposits the viral core into the cytoplasm of the target cell (Chan and Kim, 1998).

Although membrane fusion has been considered to be the predominant mechanism of HIV entry, a recent study has indicated that HIV may only enter via enodocytosis (Miyauchi et al., 2009). Previous studies had suggested that HIV endocytic entry was rare and only occurred in submucosal dendritic cells (Geijtenbeek et al., 2002). Dendritic cells present antigens to T cells in secondary lymphoid organs and they contain a specialized attachment structure called DC-SIGN (Geijtenbeek et al., 2002). HIV

Gp120 binds to DC-SIGN and then the entire intact virus is internalized within a low pH non-lysosomal endosome. The dendritic cell would then transport the intact and infective virus to the secondary lymphoid organs where the virus infected the population of T cells present there (Kwon et al., 2002). However, Miyauchi and colleagues showed using single virus imaging that HIV-1 fusion occurred in endosomal compartments and not at the plasma membrane of epithelial and lymphoid cells. Furthermore, they showed that fusion at the cell surface never went beyond the lipid mixing stage, implying that productive infections are only the result of an endocytic event.

HIV lifecycle: cytoplasmic activities

Once the viral core has been internalized the virus uncoats by shedding its viral core releasing the contents of the virus, subsequently initiating reverse transcription of the viral genome. While the process of uncoating is poorly understood, as is the degree to which reverse transcription is coupled with uncoating (Nisole and Saib, 2004), it is known that both processes involve host proteins and HIV accessory proteins. Using site directed mutagenesis it was shown that MAPK-mediated phosphorylation of Ser-109, Ser-149, and Ser-178 of p24 is necessary to facilitate proper uncoating and reverse transcription of the viral genome (Cartier et al., 1999). Additionally, a significant role for nucleocapsid as a nucleic acid chaperone has also been elucidated in these early steps; however, the presence of nucleocapsid is not an absolute requirement but likely serves to enhance the efficiency of each of these early steps, thereby vastly improving the productivity of infection (for extensive review see (Thomas and Gorelick, 2008)). Nucleocapsid has also been implicated in tRNA molecules annealing to the viral RNA to

initiate reverse transcription (Huang et al., 1998), increasing the processivity of reverse transcription by enhancing the minus strand (Peliska et al., 1994) and plus strand transfer events (Guo et al., 2000; Johnson et al., 2000), and protecting the proviral DNA from endogenous nucleases (Tanchou et al., 1998).

Nef has several functions *in vitro* for wild-type HIV production including enhancement of viral infectivity and down regulation of CD4 and major histocompatibility class I (MHC I) on the cell surface of the target cell (Schwartz et al., 1996). The mechanism of Nef enhancement in wildtype HIV infections is unknown but it has been shown is that virions produced in the absence of Nef inefficiently generate proviral DNA, although Nef does not appear to directly influence the process of reverse transcription (Schwartz et al., 1995). Interestingly, it has been shown that by pseudotyping the wildtype HIV with the VSV-G envelope protein (which permits HIV entry via the endocytic pathway) eliminates the requirement of Nef for productive infections (Aiken, 1997). It is speculated that since Nef is incorporated into wildtype HIV virions, it facilitates wildtype HIV viral core trafficking toward the endocytic machinery to aid in uncoating, a requirement that would not be necessary for HIV virions pseudotyped with VSV-G (Aiken, 1997).

The HIV accessory protein Vif was shown to be essential for proviral DNA synthesis (Fisher et al., 1987; Strebel et al., 1987) as it targets for degradation a naturally occurring intracellular inhibitor of HIV, APOBEC3G. APOBEC3G exerts an antiretroviral effect by triggering frequent G to A mutations during the process of reverse transcription, thus producing non-infective HIV virions (Harris et al., 2003). To overcome this antiretroviral effect, Vif, in the producer cell: 1) impairs the translation of

APOBEC3G (probably through an mRNA binding mechanism), 2) directly binds to APOBEC3G and recruits an E3 ubiqutin ligase to facilitate polyubiquitination and proteasome mediated degradation of APOBEC3G, and 3) inhibits APOBEC3G incorporation into newly formed virion (for extensive review see (Henriet et al., 2009)).

Reverse transcription proceeds in the cytoplasm with the *Pol* gene encoded reverse transcriptase that converts the single stranded RNA genome into a double stranded DNA copy. Viral DNA can be detected by PCR within three hours after post-infection and integrated viral DNA peaks at forty-eight hours post-infection (Butler et al., 2001). Reverse transcription is initiated using a host cell provided lysine transfer RNA ($tRNA^{Lys}$) that hybridizes to the Primer Binding Site located on the viral RNA and proceeds with a discontinuous jumping mechanism in which the RNaseH activity of RT degrades the viral RNA as it is replaced by newly synthesized DNA (Ben-Artzi et al., 1992). Reverse transcriptase lacks proof reading capabilities and consequently replication is error prone (the frequency of misincorporation by HIV-1 RT is 1 in 6,900 nucleotides polymerized on the RNA template and 1 in 5,900 on the DNA template) (Ji and Loeb, 1992). This error prone replication partly underlies HIV's ability to both evade the immune system and develop resistance to various drugs. Completion of reverse transcription yields the HIV preintegration complex (PIC). Although not every component of the PIC has been identified, it has been shown to include the double-stranded viral cDNA, integrase, matrix, Vpr, reverse transcriptase, and the host cell provided high mobility group DNA-binding cellular protein HMGA1, formerly known as HMGI(Y) (Miller et al., 1997) and the host cell provided LEDGF/p75 (lens epithelium derived growth factor) (Cherepanov et al., 2003). Although HMGA1 had been shown be

part of the PIC, it does not appear to be an absolute requirement since cell lines lacking HMGA1 show no apparent integration defects (Beitzel and Bushman, 2003). This is in contrast to LEDGF/p75 which has been shown to be an essential PIC component (Llano et al., 2006). The completed PIC then moves toward the nucleus utilizing the cellular cytoskeletal network (McDonald et al., 2002).

HIV lifecycle: nuclear entry and integration

HIV is unique among retroviruses in that it can infect non-dividing cells (Weinberg et al., 1991). In order to do this, HIV must be capable of crossing an intact nuclear membrane. Several viral proteins have been implicated in this process including matrix (Bukrinsky et al., 1993a), integrase (Gallay et al., 1997), Vpr (Heinzinger et al., 1994), and capsid (Yamashita and Emerman, 2004).

Although matrix contains two canonical nuclear targeting signals that are recognized by importins α and β ((Haffar et al., 2000), it is unclear if these signals actually facilitate nuclear import. HIV-1 virions harboring mutations within the putative nuclear targeting signals show no defects in nuclear entry (Fouchier et al., 1997) and HIV-1 virions generated in the absence of matrix were capable of nuclear import (Reil et al., 1998). Integrase also contains a nuclear import signal; however its role may not be critical for PIC nuclear translocation since disruption of the key amino acids for signaling does not alter nuclear import (Dvorin et al., 2002). Vpr contains at least three noncanonical nuclear targeting signals (Sherman et al., 2001). Vpr may facilitate nuclear entry by acting as a tether between the nuclear pore and the PIC and it has been shown that Vpr directly associates with the nuclear pore and that it binds to components of the

nuclear pore complex (Popov et al., 1998a; Popov et al., 1998b). Vpr may also facilitate nuclear entry by inducing reversible ruptures in the nuclear membrane (de Noronha et al., 2001). It is unknown if these ruptures serve as one mechanism by which the PIC transverses the nuclear membrane, however, the absolute requirement of Vpr for nuclear entry is not essential since lentiviral vectors generated in the absence of Vpr are able to infect non-dividing cells (Zufferey et al., 1997). The role that matrix, integrase, and Vpr play in nuclear import in wildtype HIV-1 infections remains controversial, and the Emerman lab has proposed that nuclear entry of HIV is not facilitated by any known combination of virally expressed proteins that possess nuclear localization signals (Yamashita and Emerman, 2005).

The Emerman lab showed that generation of chimeric HIV-1 containing the matrix and capsid of MLV lead to the loss of infectivity of non-dividing cells (Yamashita and Emerman, 2004). This suggested that either that MLV capsid negatively regulates virus infectivity in nondividing cells or that HIV capsid is an essential factor for HIV infection of nondividing cells. To determine which component was critical for infection of non-dividing cells, the Emerman lab conducted mutational analysis on the HIV capsid (Yamashita et al., 2007). It was speculated that capsid mutants which failed to infect non-dividing cells would also uncoat more slowly since there is a differential accumulation of capsid-associated MLV relative to that of HIV (Fassati and Goff, 1999; Fassati and Goff, 2001). However, this was not the case. One capsid mutant (Q63A/Q67A) behaved this way, but another (T54/N57A) actually uncoated more rapidly. Therefore, the uncoating rate of virion capsids did not correlate with the ability to transduce non-dividing cells. Additionally, these capsid mutants, as compared to

wildtype capsid, were no different in the amount of reverse transcribed product or in the kinetics of completion of reverse transcription. Even more surprising was that HIV virions with either wildtype or mutant capsid were not deficient for nuclear entry (as determined by quantification of 2LTR circle viral DNA) (Yamashita et al., 2007). Taken together, these results suggest that HIV capsid is necessary for infection of non-dividing cells, but at a critical step post nuclear entry.

Once inside the nucleus, the PIC integrates into the host genome, thereby generating a functional provirus. Although integration into the host genome is catalyzed by the HIV protein integrase, several host cell proteins aid in the process. Early studies implicated BAF (barrier to autointegration) as a critical component of the PIC in both MLV (Lee and Craigie, 1994) and HIV (Chen and Engelman, 1998). However, more recent studies have determined that a BAF knockout in mouse fibroblasts has no effect on MLV or HIV integration (Shun et al., 2007). Another early study implicated a requirement for HMGA1 (high mobility group chromosomal protein A1) for proper HIV integration (Farnet and Bushman, 1997). However, a later work showed no requirement for HMGA1 in HIV integration (Beitzel and Bushman, 2003).

Another host derived HIV co-factor that associates with integrase *in vitro* is LEDGF/p75 (lens epithelium derived growth factor) (Cherepanov et al., 2003; Maertens et al., 2003). This interaction was independently discovered by analyzing proteins associated with HIV integrase in HeLa cells (Turlure et al., 2004) and in a yeast two-hybrid screen for HIV-1 integrase interactors (Emiliani et al., 2005). LEDGF/p75 is a ubiquitously nuclear protein that tightly associates with chromatin throughout the cell cycle (Maertens et al., 2003). When endogenous levels of LEDGF/p75 were reduced

with RNAi the majority of HIV integrase relocalized to the cell cytoplasm, the integrase protein lost its chromosomal association, and there was reduced steady-state levels of integrase (Llano et al., 2004a; Llano et al., 2004b; Maertens et al., 2003). These results taken together implicate LEDGF/p75 as host cell protein essential for integrase-chromatin association, likely behaving as a receptor or molecular tether.

Integration is primarily mediated by integrase in the absence of any additional energy source in the following manner: 1) exonuclease activity trims the viral DNA to generate a two base overhang from each 3' end of the viral DNA duplex, 2) double-stranded endonuclease activity cleaves the host DNA at the integration site, and 3) ligase activity generates a single covalent linkage at each end of the proviral DNA (Bushman et al., 1990). Early analysis of HIV integrants *in vivo* and *in vitro* indicated a five base pair direct repeat of target DNA (Ellison et al., 1990; Vink et al., 1990). Later analysis has shown an additional loss of two base pairs from the ends of the donor DNA in addition to previously determined exclusive five base pair duplications of the acceptor DNA at the site of integration (Hindmarsh et al., 1999).

Initially it was thought that integration of the HIV provirus was simply a random occurrence, as opposed to other retroviruses like MLV which primarily integrated near transcriptional start sites in order to aid in their transcription (Wu et al., 2003). However, that is simply not the case. Early analysis of HIV integration sites showed a bias against centromeric alphoid repeats since these heterochromatic regions are more compact relative to euchromatic regions and these centromeric regions associate with significant numbers centromere specific proteins (CENP-A, CENP-B, and CENP-C) (Carteau et al., 1998). It was hypothesized that the tightly compacted organization of the

heterochromatin obstructed proviral cDNA integration. A later study analyzing 524 sites of HIV cDNA integration in the human genome showed that genes, and more specifically genes that were activated after infection by HIV-1, were strongly favored as integration acceptor sites (Schroder et al., 2002). Even more recently, it has been shown that LEDGF/p75, a known cellular protein that binds both DNA and HIV integrase, plays a role in controlling the location of HIV integration (Ciuffi et al., 2005). When cells were depleted for LEDGF/p75 integration occurred less frequently in transcription units, less frequently in genes regulated by LEDGF/p75, and at a higher rate in GC-rich DNA.

HIV lifecycle: proviral transcription and regulation

Once the HIV provirus is integrated into the host cell genome, the 5' LTR functions as a eukaryotic promoter. It contains upstream and downstream promoter elements including an initiator, TATA-box, and three binding sites for the transcription factor Sp1, all of which are necessary to promote transcription of the HIV transcript (Taube et al., 1999). These motifs help assemble the pre-initiation complex composed of several host proteins and position the RNA polymerase II (RNAPII) at the initiation site. Slightly upstream of the promoter is the HIV transcriptional enhancer which is bound by host cell transcription factors NFκB and NFAT (Jones and Peterlin, 1994). Stimulation of the T-cell receptor (TCR) causes NFκB to be liberated from its cytoplasmic inhibitor, IκB, and NFκB translocates to the nucleus where it induces the expression of a series of T cell activation-specific genes (Karin and Ben-Neriah, 2000). An increase in cytoplasmic Ca^{2+} levels caused by Nef in a TCR independent manner (Manninen et al., 2000) leads to NFAT dephosphorylation by calcineurin and NFAT translocation to the

nucleus where it assembles with AP1 (activator protein 1, a transcription factor) to form the fully active transcriptional complex (Crabtree, 1999). The complex containing NFAT, AP1 and NFκB as well as other cellular host proteins then dictates expression of the proviral transcript. It is worth noting that this signaling cascade for transcription activation occurs in wildtype HIV and that lentiviral vectors, which often do not require either TCR activation or possess Nef, mediate their gene expression through an internal promoter.

Once the pre-initiation complex is completely assembled, transcription proceeds from the HIV LTR. Loss of the HIV regulatory protein Tat results in a near complete failure to generate full length transcripts (Kao et al., 1987). Tat, in conjunction with the cellular protein Cyclin-T1 (CycT1), binds to a short-stem loop structure, known as the transactivation response (TAR) region, located at the 5' terminus of HIV RNAs (Kao et al., 1987). The binding of Tat to the TAR region of the HIV LTR increases transcription from the HIV LTR at least 10-fold (Marciniak et al., 1990). Tat then recruits the cellular protein cyclin-dependent kinase 9 (Cdk9) to the HIV LTR (Wei et al., 1998). Cdk9 phosphorylates the carboxylterminal domain of RNAPII, thereby converting transcription from an initiation phase to an elongation phase (Price, 2000) and HIV transcription can progress. Transcription of the integrated viral genome results in greater than a dozen different HIV transcripts (Saltarelli et al., 1996). Some transcripts remain in the nucleus in unspliced forms, some are processed as they are transcribed, and others are transported to the cytoplasm for protein expression (Cullen, 1998).

One completely spliced message that is exported out of the nucleus for immediate translation is the transcript encoding the accessory protein Rev. This protein is essential

for regulation of the export of incompletely spliced or unspliced RNA from the nucleus to the cytoplasm (Cullen, 1998). Rev binds to a 240-base region of complex RNA secondary structure in the second intron of HIV called the Rev Response Element (RRE) (Cullen, 1998; Malim et al., 1989). The binding of Rev to the RRE facilitates the export of unspliced and incompletely spliced viral RNAs from the nucleus to the cytoplasm when normally only completely spliced messages are exported out of the nucleus (Cullen, 1998). Rev expression, and consequently the regulation of export of unspliced or partially spliced messages, is regulated by a negative feedback loop (Felber et al., 1990). High levels of Rev expression can lead to the export of so much unspliced viral RNA that the amount of spliced RNA available for translation is decreased which leads to a reduction in Rev protein expression as Rev is only expressed from spliced message (Felber et al., 1990).

Rev functions in conjunction with several host cell proteins. Rev contains both a nuclear localization signal (NLS) and a nuclear export signal (NES) (Cullen, 1998). This NES interacts with CRM-1 (an NES receptor) and exportin-1 (a shuttling protein). The export complex that is formed between Rev, CRM-1, exportin-1, and the viral RNA also requires another cellular protein, RanGTP (Cullen, 1998). The Ran nucleotide exchange factor, RCC1, which charges Ran with GTP, is localized predominantly in the nucleus while the GTPase activating protein specific for Ran (RanGAP) is localized in the cytoplasm (Moore, 2001). Cargo leaving the nucleus is only effectively loaded onto CRM1/exportin-1 in the presence of RanGTP (Cullen, 1998). When this complex reaches the cytoplasm, the GTP is hydrolyzed and the viral RNA cargo is released. HIV virion production requires a delicate balance between export of spliced and unspliced

message. If splicing is too efficient, then only the multiply spliced transcripts, which are insufficient to support full viral replication, are transported to the cytoplasm. However, if splicing is impaired, adequate synthesis of Tat, Rev, and Nef does not occur and once again viral replication will be inhibited. This critical balance is essential for proper HIV replication (Malim and Cullen, 1993). Rev and Tat play an integral role in the regulation of viral RNA and their proper regulation is necessary for HIV to complete its lifecycle.

HIV lifecycle: replication and assembly

To ensure the proper cytoplasmic environment for wildtype HIV replication various accessory proteins "prime" the intracellular environment. One HIV accessory protein, Nef, plays a critical role in optimizing the cytoplasmic components for efficient replication. Nef is necessary for the progression of HIV to AIDS and HIV species which lack Nef have a reduced rate of disease progression (Deacon et al., 1995; Kestler et al., 1991). Nef interacts with the PI3K (Phosphoinositide 3-kinase) cascade and induces remodeling of the intracellular actin network, causing lipid raft movement and the formation of larger raft structures that have been implicated in alterations of normal T-cell receptor signaling (Geyer et al., 2001; Skowronski et al., 1993). Since Nef heavily influences the formation of lipid rafts its not surprising that Nef and other viral structural proteins have been shown to colocalize with lipid rafts (Wang et al., 2000; Zheng et al., 2001).

A second important function of Nef is to help the infected host cell avoid detection by the immune system in order to maximize virus production. Nef does this in two interrelated ways. First, Nef impairs the immunological response to HIV by

activating FasL (fas ligand), which induces apoptosis within the neighboring Fas expressing cytotoxic T cells that could destroy HIV positive cells (Xu et al., 1999). Nef also reduces the expression of surface MHC I in the infected cell thereby decreasing the possibility of being recognized by CD8 cytotoxic T cells (Collins et al., 1998). Nef is also capable of maximizing virus production by inhibiting apoptosis in two ways. First, Nef binds and inhibits ASK-1 (apoptosis signal regulating kinase-1) that is involved in both Fas and TNFR (tumor necrosis factor receptor) signaling (Wolf et al., 2001). Second, Nef binds the tumor suppressor protein p53 thereby inhibiting another initiator of apoptosis (Greenway et al., 2002). Through its activities, Nef maximizes viral reproduction.

Once the right balance of viral transcripts have been synthesized and translated, viral assembly occurs. The first major player in viral assembly is the Gag precursor polyprotein Pr55. Pr55 contains signal sequences which: 1) target and bind it to the plasma membrane, 2) promote Gag-Gag interactions, 3) encapsidate dual copies of the RNA genome (via the Ψ "packaging" sequence found on the 5' end of the RNA (Berkowitz et al., 1996)), and 4) stimulate budding from the cell (Freed, 2001). The Pr55 is ultimately cleaved by the HIV protease during the maturation stage of the virus and leads to the production of the p17 MA, p24 CA, p7 NC, p6, and two spacer peptides, SP1 (originally called p2) and SP2 (originally called p1) (Murakami, 2008). The second major player in viral assembly is the Env protein, a synthesized Gp160 precursor which is glycosylated and oligomerized in the endoplasmic reticulum and ultimately processed into Gp120 and Gp41 by a host protease and transported to the cell membrane for incorporation into virions (Murakami, 2008). It is likely that lipid rafts (Ono and Freed,

2001) or tetraspanin enriched microdomains (Jolly and Sattentau, 2007; Nydegger et al., 2006) serve as scaffolds within the plasma membrane to facilitate the interactions necessary to complete assembly and budding. One protein, ABCE1 (formerly HP68 or RNase L inhibitor), serves as a molecular chaperone, facilitating conformational changes in Gag required for viral capsid assembly (Zimmerman et al., 2002).

HIV lifecycle: budding and maturation

The final step in the life cycle of HIV that is still tied to the infected cell is the process of budding. The budding reaction involves the action of several proteins, including the "late domain" sequence (PTAP) present in the p6 portion of Gag (Garnier et al., 1999). Ubiquitination may also play a role as the product of the tumor suppressor gene 101 (TSG101), a homologue of the Ubc4 class of ubiquitin conjugating E2 enzymes, was shown to bind to the PTAP motif of p6 and appears to be essential for proper HIV budding (Garrus et al., 2001; VerPlank et al., 2001). Normally TSG101 acts in the vacuolar protein sorting pathway that selects cargo to be dumped into the multivesciular body (MVB) and the MVB fuses with lysosomes to degrade the contents of the MVB (Katzmann et al., 2001). MVBs are usually produced when surface patches on late endosomes bud away from the cytoplasm into the lumen of the endosome (Morita and Sundquist, 2004). It appears that HIV has hijacked this intracellular pathway to aid in its own budding.

The HIV accessory protein Vpu also appears to play a critical role in budding as in the absence of Vpu large numbers of virions are seen attached to the surface of infected cells (Klimkait et al., 1990). This role was recently defined in a series of papers

which determined that bone marrow stromal cell antigen 2 (BST-2, also known as tetherin) inhibits the release of retrovirus particles in certain cells and this inhibition can be overcome by Vpu (Neil et al., 2008; Van Damme et al., 2008). *Bst-2* expression correlated with a requirement for Vpu during HIV-1 and MLV virus particle release and in cells where HIV-1 virion release required Vpu expression, depletion of BST-2 eliminated this requirement (Neil et al., 2008). BST-2 contains both an N-terminal transmembrane domain and a C-terminal glycosyl-phosphatidylinositol (GPI) anchor thereby localizing BST-2 to both the cell surface and intracellular region. Additionally, BST-2 can be efficiently internalized from the cell surface (Kupzig et al., 2003). It was postulated that this unique structure, coupled with the fact that BST-2 associates with cholesterol-enriched lipid rafts (Kupzig et al., 2003), may allow BST-2 to tether enveloped virions to the cellular membrane and inhibit the release of these virions (Van Damme et al., 2008). However, a recent study which was unable to detect the presence of BST-2 on virus particles that were mechanically sheared from the cell surface casts doubt on the role of BST-2 as tethering protein (Miyagi et al., 2009). The authors suggested that miniscule amounts of BST-2 could be below their level of detection, but that other models of the mechanism of action of BST-2 and Vpu must be considered.

HIV maturation is coupled to virion release from the cell surface. The HIV protease initiates maturation by proteolytically processing the Gag and Gag-Pol polyproteins. The HIV protease found within the immature virion cleaves Gag (Pr55) into the p17 MA, p24 CA, p7 NC, p6, and two spacer peptides, SP1 and SP2 (Schubert et al., 2000). The Pol portion of the Gag-Pol is cleaved into the protease, reverse transcriptase, and the integrase enzymes (Vogt, 1996). If these proteolytic processing

steps are disrupted or altered, virus particles are generated with aberrant morphology and reduced infectivity (Kaplan et al., 1993). This indicates that maturation is a critical step in order to generate infective virus particles.

HIV Therapies

The development of current therapies

HIV proteins and their known functions are summarized in Table 1-1. Most current therapies currently target one or more of these known functions. One long-term solution to combat HIV would be to develop a vaccine, however HIV's propensity toward quick evolution and latency severely restricts this possibility (Walker and Burton, 2008). Currently, these antiretroviral drugs are not administered singly to HIV positive patients but are administered in combinations in a specific program that has been dubbed highly active antiretroviral therapy (HAART). The concept of drug combination therapy was first developed the late 1980s when it was determined that a combination of drugs lead to a synergistic inhibition of human T-cell leukemia virus (Hartshorn et al., 1986). Eventually this combinatorial approach was applied to HIV, resulting in a synergistic suppression of the virus using the first two United States Food and Drug Administration (FDA) approved antiretroviral drugs, zidovudine (AZT) and didanosine (dideoxyinosine; ddI) (Dornsife et al., 1991). This application came at a very opportune time as just two years earlier it was determined that prolonged exposure to AZT alone resulted in HIV with reduced sensitivity to the drug (Larder et al., 1989). Larder and colleagues showed that HIV could quickly become drug resistant, and therefore a combination of highly

Table 1-1: HIV Proteins and their Functions

Protein	Gene Source	Function
Matrix	Gag	Structural protein implicated in uncoating, PIC formation, and nuclear entry
Capsid	Gag	Structural protein that encapsidates the viral RNA and catalytic proteins in the mature virus
Nucleocapsid	Gag	Structural protein that serves as a nucleic acid chaperone, promotes reverse transcription processivity, and protects the viral RNA from degradation by endogenous nucleases
p6	Gag	Structural protein that facilitates viral budding and stabilizes the structure of the mature virus
Reverse Transcriptase	Pol	Catalytic protein that converts the single stranded viral RNA to the double stranded provirus suitable for integration, contains RNase H activity
Integrase	Pol	Catalytic protein responsible for the integration of the provirus into the host genome using several host proteins
Protease	Pol	Catalytic protein responsible for the cleavage of various polyproteins generated from Gag, Pol, and Env
Gp120	Env	Surface portion of the envelope that mediates CD4 and co-receptor mediated binding
Gp41	Env	Transmembrane portion of the envelope that mediates fusion between the virus and host cell
Nef	independent	Accessory protein that downregulates surface CD4, inhibits apoptosis, and aids the host cell in avoiding the immune system
Rev	independent	Accessory protein that binds the Rev Response Element on viral transcripts to facilitate the nuclear export of incompletely spliced RNA
Tat	independent	Accessory protein that binds TAR and along with CycT1 and CDK9 enhances RNAPII elongation of viral template
Vif	independent	Accessory protein that targets naturally occuring antiviral APOBEC3G for degradation
Vpr	independent	Accessory protein found in the PIC and implicated in nuclear entry
Vpu	independent	Accessory protein necessary for budding as it downregulates CD4 and BST-2

Table 1-1

HIV Proteins and their functions.

The proteins encoded by the HIV virus, where they are derived from, and their known and implicated functions.

potent drugs, acting on different proteins, in optimized combinations, would be necessary to combat the disease.

Current therapies: antiretrovirals

So far, all the drugs that have been developed to combat HIV specifically target a function of HIV. Although other drugs have affected HIV by altering the immune system, no studies have proven if any of these drugs actually provide a clinical benefit by prolonging a patient's life. For example, BAY 50-4798, a recombinant interleukin-2 homolog, was used in combination with HAART which resulted in no sustained immunological effect (Davey et al., 2008). Another immunological effector, hydroxyurea, was shown to inhibit HIV replication and was thought to be promising because it targeted T-cells instead of HIV and therefore resistance would be slow to acquire if at all (Lori et al., 1994). However, long term clinical studies have generated mixed results (Gelone and Kostman, 1999), and the consensus opinion is that hydroxyurea treatment must be monitored closely given its highly toxic side effects (Lori et al., 2005; Swindells et al., 2005). Since non-HIV based drugs have failed to generate positive results, almost all the newly developed drugs have been retroviral specific.

The antiretroviral drugs used in HAART fall into the following general categories: nucleoside reverse transcription inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (nNRTI), protease inhibitors (PI), early inhibitors (including fusion inhibitors), integrase inhibitors, and most recently maturation inhibitors (Table 1-2, for review see (Bailey and Fisher, 2008)). The first antiretroviral drugs developed

Table 1-2: Categories and Examples of Current HIV Drugs

Drug Type	Mechanism of Action	Examples
NRTI	Inhibits viral DNA synthesis by serving as a nucleotide analog	emtricitabine, amivudine, zidovudine, didanosine, stavudine, abacavir
nNRTI	Inhibits viral DNA synthesis by directly binding to RT	etravirine, delavirdine, efavirenz, nevirapine
Protease Inhibitors	Suppresses the activity of HIV protease thereby inhibiting final assembly	amprenavir, tipranavir, indinavir, saquinavir, ritonavir, atazanavir, nelfinavir
Integrase Inhibitors	Prevents HIV integrase from integrating the viral genome into the host	raltegravir, elvitegravir (in clinical trials)
Early Inhibitors	Binds up the HIV co-receptor CCR5 preventing virus entry	maraviroc, vicriviroc (experimental), PRO 140 (experimental)
Fusion Inhibitors	Blocks gp41 and prevents the conformation change necessary for fusion	Enfuvirtide
Maturation Inhibitors	Binds p25 Gag protein and prevents its breakdown to p24 and SP1	Bevirimat

Table 1-2

HIV drugs.

HIV drugs fall into seven different categories based on their mechanism of action. The first drugs that were developed were NRTIs (nucleoside reverse transcription inhibitors, nNRTIs (non-nucleoside reverse transcription inhibitors), and protease inhibitors and therefore they have the most examples of drugs currently on the market.

were in the category of NRTI and the first drug zidovudine (AZT) was approved by the FDA over 20 years ago (Kolata, 1987). NRTIs inhibit viral DNA synthesis by serving as nucleotide analogs that terminate DNA synthesis while nNRTIs inhibit viral DNA synthesis by directly binding to the reverse transcriptase thereby interfering with its function. PIs target viral assembly and maturation by suppressing the activity of HIV protease. Integrase inhibitors block the function of integrase thereby preventing the viral genome from integrating into the host genome. Entry/fusion inhibitors either bind the HIV co-receptor CCR5 preventing virus entry or block Gp41 and prevent the conformational change necessary for viral and host cell fusion. Most recently, the maturation inhibitor PA-457 (Bevirimat) was developed that inhibited anti-HIV activity by targeting a late step in Gag processing (Li et al., 2003). Unlike PIs, this inhibitor did not bind to the protease but specifically bound to the p25 Gag protein and prevented its breakdown to p24 and SP1/p2 (Salzwedel et al., 2007). The development of antiretrovirals has reshaped how people live with HIV and the current drug mixes make living with HIV "manageable." However, if we wish to ultimately control or eradicate HIV better drugs will be necessary or ideally, a vaccine.

Current therapies: long-term ramifications

The application of HAART has dramatically reduced HIV mortality and increased quality of life for those infected (Pomerantz and Horn, 2003). First line treatment under HAART consists of two NRTIs and either a PI or an nNRTI (Bailey and Fisher, 2008). Although this is the gold standard HIV regimen and can reduce plasma viremia to clinically undetectable levels (Gulick et al., 1997), approximately 25% of patients do not

achieve viral suppression or lose it within two to three years (Touloumi et al., 2006). Additional long term benefits from HAART are hampered by several factors including adherence to a complicated medicine dosage schedule (Paterson et al., 2000), undesirable side effects (Carr and Cooper, 2000), and long term viral latency within HIV reservoirs that make complete eradication of the virus a near impossibility (Geeraert et al., 2008). However, the biggest problem facing long-term HAART therapy is the development of drug resistant HIV. The current antiretroviral therapies exert enormous selection pressures on HIV leading to the emergence of resistant strains (Coffin, 1995). HIV is perfectly equipped for speedy evolution given its rapid replication rate (up to 10^{10} new viruses generated and cleared in one day in untreated individuals) (Perelson et al., 1996) and error prone reverse transcriptase (an error being introduced every 10^4 to 10^5 nucleotides, which translates to approximately once per viral genome) (Mansky and Temin, 1995). Although resistant HIV tends to develop in individuals who have already been subjected to an initial round of failed HAART, resistance can also appear during the first round of HAART (Richman et al., 2004). Since resistant HIV is becoming more and more prevalent, the occurrence of transmitted HIV resistance is also on the rise (Booth and Geretti, 2007). Even with newer and more powerful drugs, HAART is simply a stop-gap solution to the HIV pandemic and in order to eradicate HIV new strategies will be necessary.

Retroviral Vectors

Retroviral Vectors: History

Given how easily retroviruses can exploit the cellular machinery and integrate within the host cell genome it is not surprising that they have been harnessed by researchers to stably deliver genetic material into cells for almost 30 years (Wei et al., 1981). The first virus to be utilized in this manner was the Moloney Murine Leukemia Virus (MLV). MLV was chosen since researchers were familiar with this virus and already possessed sufficient tools to manipulate it. One early drawback to using MLV was that, on occasion, it lead to the creation of replication competent retroviruses (RCRs) (Donahue et al., 1992; Mann et al., 1983; Otto et al., 1994; Vanin et al., 1994). Early on, researchers realized that the RCRs would inhibit their ability to use retroviral vectors in further human genetic as well as gene therapy studies, so they began to artificially engineer a safer virus. Second generation MLV retroviral vectors were created by generating split-genome plasmids: the first plasmid (pMSVgpt) containing the MLV viral vector expressing a selectable marker, no viral proteins, and a packaging sequence (Ψ) and the second "helper" plasmid deficient for the packaging sequence (pMOV-Ψ^{-}) containing the *Gag*, *Pol*, and *Env* genes (Mann et al., 1983). However, even with this advancement a simple recombination event between the two plasmids could generate low levels of wildtype RCRs (Mann et al., 1983; Miller and Buttimore, 1986). To further prevent the generation of RCRs, third generation retroviral vectors were created with triply split genome plasmids: one encoding the MLV retroviral vector, one encoding the *Env* gene, and another encoding the *Gag*/*Pol* genes (Markowitz et al., 1988). These third generation MLV retroviral vectors would require multiple plasmid recombination events

to generation an RCR. Even with this level of engineering, RCRs were still detected at very low levels (Chong et al., 1998; Chong and Vile, 1996).

Retroviral vectors: Development of lentiviral vectors

The HIV genome is organized so that all HIV proteins can be generated from variantly spliced mRNAs (Fig. 1-1). HIV was developed into a gene delivery mechanism when it was shown that it could infect non-dividing cells (Bukrinsky et al., 1993a). However, before HIV could be used as a gene delivery tool, significant effort was expended to engineer wildtype HIV into a lentiviral vector. Initially, because so little was known about the accessory proteins, virus propagation was very difficult (reviewed in (Buchschacher and Wong-Staal, 2000)). The first method utilized to convert HIV into a lentiviral vector was to simply to disrupt the HIV *Env* gene and then provide that *Env* gene *in trans* (Helseth et al., 1990; Page et al., 1990). This system did generate lentiviral vectors, but titers were low and by using the wildtype HIV *Env*, the retroviral vectors were only capable of infecting CD4+ cells (Helseth et al., 1990; Page et al., 1990).

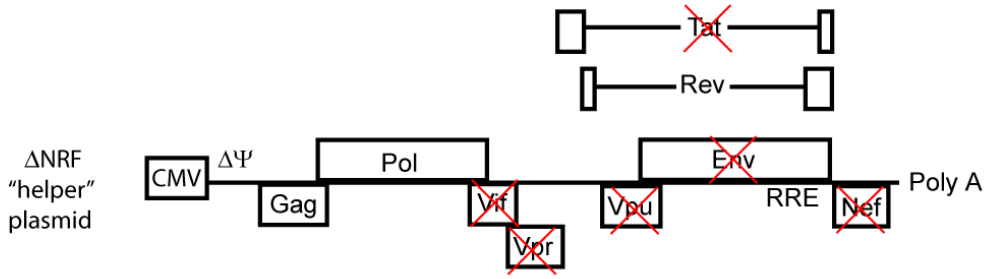
Eventually, a first generation lentiviral vector system was created (Naldini et al., 1996). In order to accomplish this Naldini and colleagues first substituted the vesicular stomatitis virus glycoprotein envelope (VSV-G) for the HIV Gp160 envelope. It had been shown previously in MLV retroviral vectors that pseudotyping with this envelope greatly expanded the range of host targets (Yee et al., 1994). Furthermore, Naldini and colleagues divided the lentivirus genes between three separate plasmids and used a simple expression construct with a CMV promoter and poly-A tail to transcriptionally regulated the expression of the *Env* and *Gag/Pol* components. Since replication

competent lentiviruses (RCLs) were still a concern, second generation lentiviral vectors simply deleted additional HIV accessory proteins (Vif, Vpu, Nef, and Vpr) without any loss in infectivity (Zufferey et al., 1997). To further prevent the generation of RCLs, third and fourth generation lentiviral vectors were developed. Third generation vectors contained two improvements: 1) creating a chimeric 5' LTR that replaced the U3 region with the immediate early cytomegalovirus enhancer/promoter eliminating the need for HIV Tat protein and 2) the *Rev* gene was removed from the packaging plasmid and placed on its own plasmid (Dull et al., 1998). By replacing the U3 region in the 5' LTR with the CMV enhancer promoter it was possible to generate a safer self-inactivating (SIN) vector. SIN vectors had already been developed and used in MLV retroviral vectors but SIN vectors typically yielded poor titers (Hwang et al., 1997; Yu et al., 1986). However, when lentiviral vectors were made self-inactivating no loss in titer or infective potential was seen (Miyoshi et al., 1998). Ultimately it was shown that these fourth generation SIN lentiviral vectors cannot produce RCLs even if the target cell is already infected with wildtype replication competent HIV (Bukovsky et al., 1999). A diagram of a third generation lentiviral vector system is presented in Fig. 1-4.

Generating high titer lentiviral vectors

The most common laboratory based method to generate high titer lentiviral vector is to utilize the calcium phosphate transfection system initially developed in 1973 (Graham and van der Eb, 1973) and subsequently optimized with a new variation of the buffer (Chen and Okayama, 1987). In this system the lentiviral vector plasmid and all split genome catalytic, structural, accessory proteins, and a viral envelope plasmid are


Lentiviral Vector Plasmid
CMV
R
U5
Ψ
cPPT
EF1α
Gene of Interest
ΔU3
R
U5


Tat
Rev
ΔNRF "helper" plasmid
CMV
ΔΨ
Gag
Pol
Vif
Vpr
Vpu
Env
RRE
Nef
Poly A

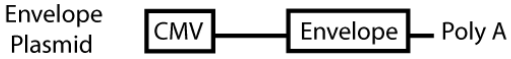

Envelope Plasmid
CMV
Envelope
Poly A

Figure 1-4

Organization of lentiviral vector plasmids.

In order to generate lentiviral vectors without the possibility of creating replication competent lentivirus, the viral genome was split into three plasmids, the vector was made self-inactivating, various accessory proteins were deleted, and an alternative envelope was used. The lentiviral vector plasmid generates a self inactivating virus and is tat independent because of alterations to the LTRs. The ΔNRF helper plasmid contains only the catalytic, structural, and accessory proteins necessary to create a retroviral vector and eliminates nonessential proteins. The Envelope plasmid does not encode for Gp120/Gp41, but instead codes for any other envelope (usually, the vesicular stomatitis G-protein or VSV-G envelope).

transfected into a cell line (usually 293T since they are highly amenable to this method of transfection) and the lentiviral vector buds out into the supernatant to be collected seventy-two hours later (Fig. 1-5). Typically after viral vector concentration this methodology can yield titers approaching 10^9 infectious units/milliliter (IU/ml) (Kafri et al., 1999). However in order to become a true tool for clinical applications, scalability is necessary. Therefore, a packaging cell line was needed that could stably produce high titer virus while at the same time eliminate all possibility of RCLs. Early attempts resulted in low viral titer (10^4 IU/ml) that could only infect CD4+ cells (Corbeau et al., 1996; Yu et al., 1996). The use of VSV-G as the envelope protein increased the possibility of creating a high titer packaging cell line. However, since VSV-G is toxic at high concentrations due to its ability to fuse membranes between cells (Li et al., 1993), the ideal cell line would need to be inducible. The first tetracycline inducible system produced unconcentrated titers at 10^6 IU/ml, was able to infect both dividing and non-dividing cells, and did not produce any detectable levels of RCLs (Kafri et al., 1999). This packaging cell line was produced from first generation lentiviral vector plasmids, and improvements have lead to the creation of a packaging cell line based on fourth generation split genome SIN vectors (Xu et al., 2001).

Uses of retroviral vectors: gene therapy

Given that both MLV retroviral vectors and lentiviral vectors are capable of integrating into the genome of a target cell and producing long-term gene expression (Fig. 1-6), the first logical application was in the realm of gene therapy. Gene therapy covers a broad spectrum of applications, including gene replacement and knockdown for

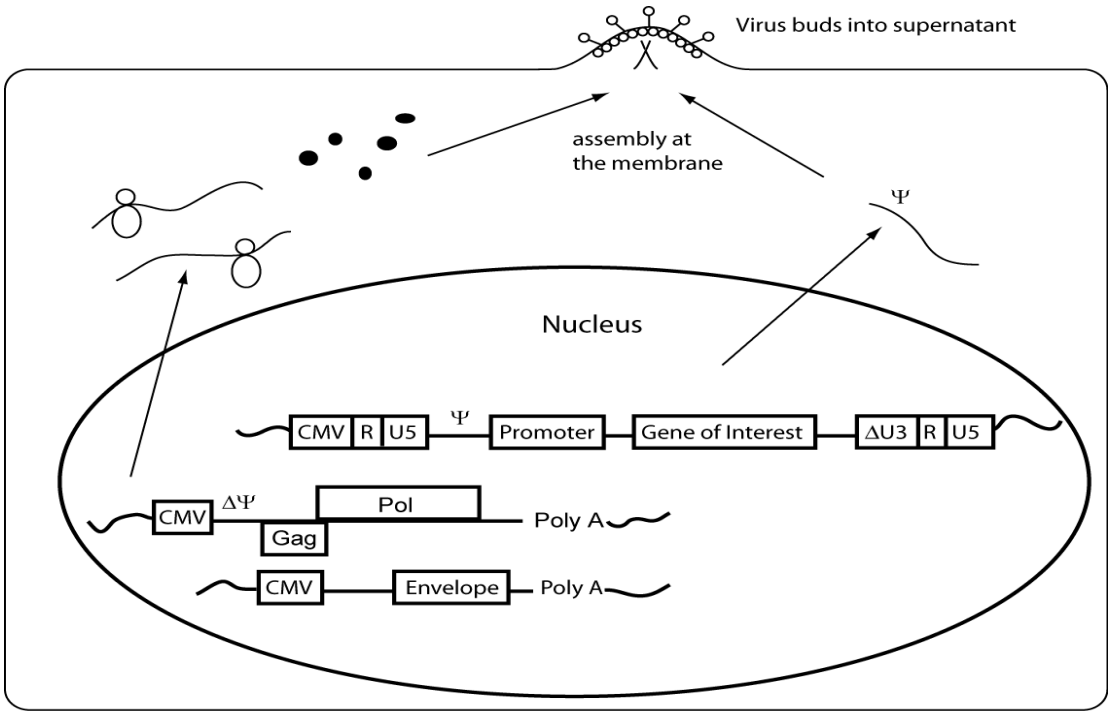

Virus buds into supernatant
assembly at
the membrane
Ψ
Nucleus
CMV
R
U5
Ψ
Promoter
Gene of Interest
ΔU3
R
U5
CMV
ΔΨ
Pol
Gag
Poly A
CMV
Envelope
Poly A

Figure 1-5

Generating high titer lentiviral vector.

The lentiviral vector plasmid, the helper plasmid, and the envelope plasmid are transfected into a packaging cell line (usually 293T cells). The DNA constructs are transcribed and translated into proteins necessary to create a lentiviral vector. The proteins assemble into a lentiviral vector at the plasma membrane and the virus buds out into the supernatant where it can be collected.

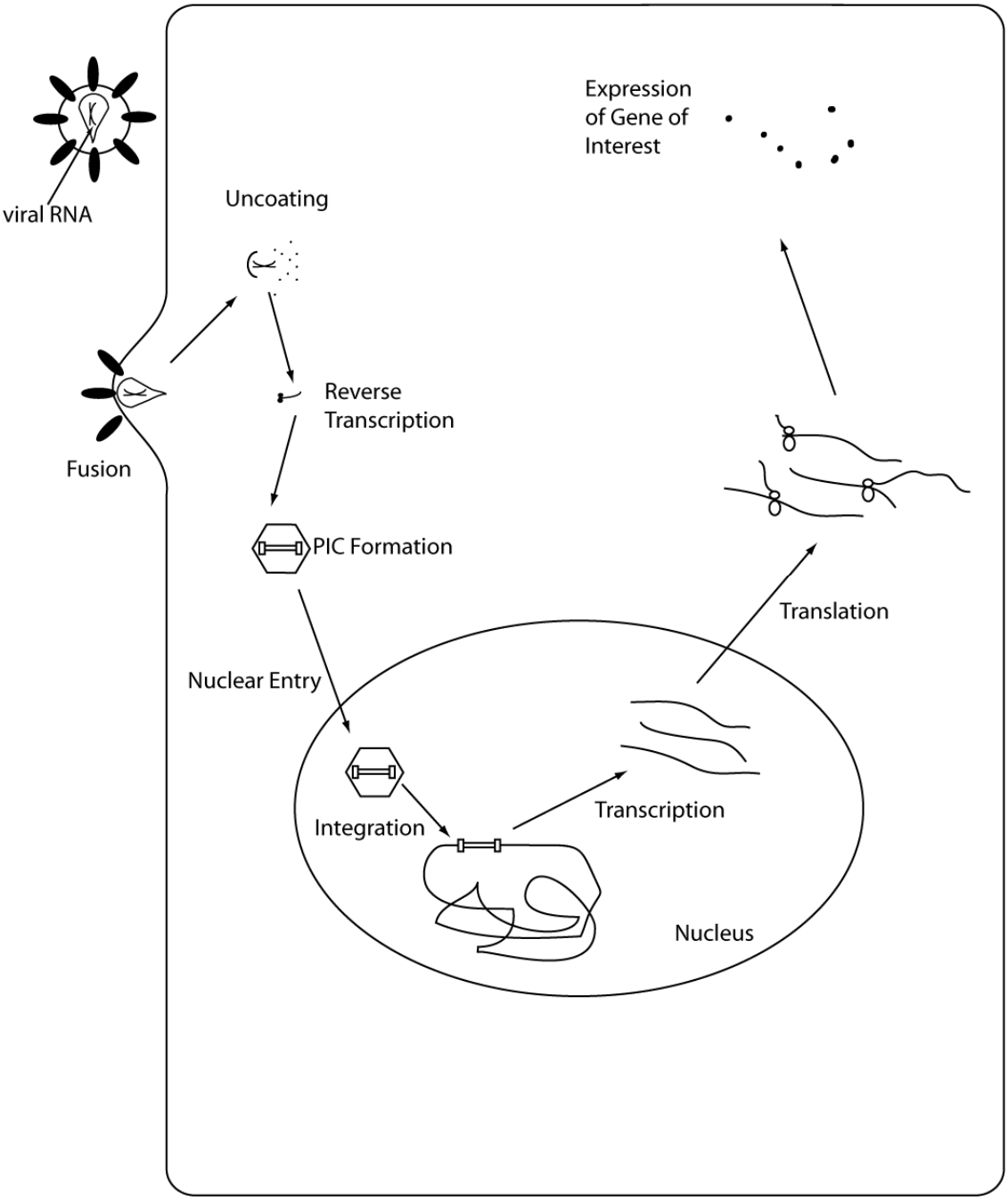

viral RNA
Uncoating
Expression
of Gene of
Interest
Fusion
Reverse
Transcription
PIC Formation
Translation
Nuclear Entry
Integration
Transcription
Nucleus

Figure 1-6

Lentiviral vector infective pathway.

Lentiviral vectors recapitulate the early lifecycle of wildtype HIV. After entry and once in the cytoplasm, the lentiviral vector uncoats, reverse transcribes its genome, and forms a preintegration complex (PIC). The PIC crosses the nuclear membrane, integrates into the host genome, and initiates transcription of the viral genome. The viral RNA, which encodes a gene of interest, is transported out into the cytoplasm where the gene of interest is translated and the protein is expressed.

genetic or acquired diseases. Replacement gene therapy can be conducted at the germ cell level (although this is currently highly controversial and limited in nearly all jurisdictions because of the ethical ramifications) or at the somatic cell level. The most common form of replacement gene therapy has been therapeutic cell gene therapy. Therapeutic gene therapy places a normal gene at an ectopic location within the genome to replace a non-functional copy, and this approach has been utilized in both *ex vivo* and *in vivo* applications (for extensive review see (Rubanyi, 2001)). Suicide gene therapy attempts to destroy a certain population of cells by transducing them with a gene that codes for a toxic product either directly or by converting a non-lethal prodrug into a toxic product (Mancheno-Corvo and Martin-Duque, 2006). Suicide gene therapy has been used extensively to target cancer (Hughes, 2004).

The first significant milestone in clinical retroviral gene replacement therapy in humans was the use of MLV to correct the SCID-X1 defect by *ex vivo* gene transfer into CD34+ cells (Cavazzana-Calvo et al., 2000). A similar study conducted in London was also able correct this defect (Gaspar et al., 2004). The degree of success was reduced when it was shown that several of the patients acquired leukemia due to a retroviral insertion event close to the *LMO2* proto-oncogene promoter (Hacein-Bey-Abina et al., 2003). Of the twenty patients with X-linked SCID treated in these two trials, 18 are currently alive with good immune reconstitution, but five of the twenty have experienced a serious side effect, leukemia. Of these five patients, three are in remission, one died of therapy related leukemia, and one died of complications of a subsequent stem cell transplant because of the failure of the first gene therapy attempt (Nathan and Orkin,

2009). Investigators are currently determining what caused this adverse side effect and how to avoid it in the future (Strauss and Costanzi-Strauss, 2007; Yi et al., 2005).

Suicide gene therapy has been applied in the clinical setting to destroy cancer cells *in vivo*. This therapy has commonly used MLV since MLV can only integrate into actively dividing cells and cancer cells are among the most actively dividing cells in the body. Several studies have been carried out using different genes to target cancer cell destruction. In an early study, p53 was used to induce apoptosis in lung cancer tumors with no evidence of toxicity (Roth et al., 1996). Although this study did not indicate any clinical improvement in the patients because of inefficient retroviral delivery, it did provide proof of the principle that p53 could induce apoptosis *in vivo*. Several studies have used herpes simplex virus thymidine kinase (HSV-*TK*) in conjunction with its prodrug gancyclovir to induce cell death. The advantage of this prodrug approach is two-fold: 1) the gene itself is not toxic to the cells and 2) it utilizes the bystander effect whereby non-transduced cells are also killed via diffusion of the cytotoxic product (Moolten, 1986). Two HSV-*TK* studies using retroviral vectors have been used to target a type of brain cancer, glioblastoma, with positive results (Sandmair et al., 2000; Shand et al., 1999). HSV-*TK* along with another suicide gene, cytosine deaminase, has also been used to treat breast cancer with an observable reduction in tumors (Takahashi et al., 2006).

siRNA and HIV

Background: RNAi and HIV

The RNAi (RNA interference) phenomenon utilizing siRNA (small interfering RNA) to induce posttranscriptional gene silencing is a relatively recent discovery (Fire et al., 1998). The mechanism of action is being elucidated and it is quickly becoming another valuable tool for the researcher. For extensive explanation and review of the mechanism of siRNA activity and different classes of siRNAs see (Matranga and Zamore, 2007) and (Chapman and Carrington, 2007). At its most basic level, RNAi is mediated by a small RNA that exerts an inhibition of gene expression at the posttranscriptional level based on sequence homology between the siRNA and the mRNA. As further investigation was conducted it soon became apparent that RNAi served a variety of functions *in vivo* besides knockdown of translation. It has been shown that RNAi suppresses transposon mobilization in *Caenorhabditis elegans* (Ketting et al., 1999) and that it plays a role as an endogenous host cell defense against viruses in plants (Waterhouse et al., 2001). Taken together these two results suggest a potential role of RNAi in human/virus interaction.

One potential role of RNAi in host cell-HIV interactions has been elucidated by identifying virally produced miRNAs (micro RNAs) that target viral or host cell RNA. The results generated in these studies are highly controversial since some believe that HIV does not produce any viral miRNAs (Lin and Cullen, 2007). Several groups have shown that HIV does encode miRNAs that are used to regulate viral RNAs. One group of investigators identified LTR and Nef specific miRNAs that regulate HIV RNA transcription (Omoto and Fujii, 2005; Omoto et al., 2004). Even more recently, two

groups have identified an miRNA that is derived from the TAR region of HIV that regulates HIV RNA expression (Klase et al., 2007; Ouellet et al., 2008). The TAR region, which is a hairpin structure found on the 5' end of HIV transcripts and is essential for Tat mediated transactivation of viral gene expression, was shown to interact with the endogenous Dicer protein that cleaves the hairpin and generates the miRNA. Although no HIV generated miRNAs have been detected experimentally, potential HIV encoded miRNAs have been identified via sequence analysis and computer based folding models (Bennasser et al., 2006; Couturier and Root-Bernstein, 2005). Evidence suggests that HIV encoded miRNAs do exist and they target both the regulation of HIV RNA and cellular RNA in order for HIV to complete its lifecycle.

A second category of RNAi based host cell-HIV interactions is the identification of host miRNAs targeting viral RNA or host RNA utilized by the virus. One study has shown that overexpression of certain miRNAs in quiescent T4 lymphocytes specifically targets the 3' end of HIV RNA, silencing virtually all of the viral message (Huang et al., 2007). Another computer based study identified five miRNAs expressed in T-cells that could target Nef, Vpr, Vif, and Env RNAs (Hariharan et al., 2005). Additionally, suppression of the miRNA pathway proteins Dicer and Drosha leads to greater infectivity possibly implicating a role for these proteins in inhibiting HIV infections (Triboulet et al., 2007). It was also shown in this study that HIV infections cause an upregulation of some miRNAs and a downregulation of others. Two cellular miRNAs, miR-17-5p and miR-20, are upregulated upon infection and specifically silence the mRNA encoding the histone acetylase PCAF. PCAF had previously been shown to promote Tat transactivation by remodeling the histones near the LTRs thereby promoting HIV gene

expression (Kiernan et al., 1999). This secondary observation indicates that productive infections by HIV could activate miRNA based cellular host defenses that suppress host RNAs necessary for viral gene expression.

Applications of Retroviral Vectors

Uses of retroviral vectors: RNAi therapy for HIV infections

One of the first targets for retroviral based siRNA therapy was HIV itself. siRNAs were designed against multiple HIV transcript regions including Gag, Pol, Env, Vif, Vpr, and the LTR and these siRNAs all resulted in downregulation of the HIV transcript in cell lines (Coburn and Cullen, 2002; Jacque et al., 2002; Lee et al., 2002). Although these early studies showed that HIV infection could be modulated by siRNAs that target the HIV transcripts for degradation, it quickly became apparent that clinical applications were still very far away. When RNAi was applied to cells infected with HIV it triggered extremely high mutation rates. When this was coupled to the fact that RNAi required very tight binding between the siRNA and the target transcript, this lead to the development of HIV escape mutants that easily evaded transcript degradation. In some cases, HIV evolved an alternatively folded RNA structure which made RNAi less efficient (Westerhout et al., 2005), but in the majority of cases HIV simply evolved point mutations that prevented tight siRNA binding (Boden et al., 2003; Gitlin et al., 2002). Although the use of siRNA to downregulate HIV infections worked in principle, alternative strategies are needed to take the technique from the laboratory to the clinic.

One approach to avoid the problem of HIV overcoming RNAi mediated transcript degradation is to target cellular transcripts that are necessary for HIV entry and

replication. Cellular cofactors such as NFκB, HIV receptor CD4, and the co-receptors CCR5 and CXCR4 have all been downregulated, leading to a block in viral replication or entry (Anderson and Akkina, 2005; Novina et al., 2002; Surabhi and Gaynor, 2002). Although targeting of cellular proteins in this manner may not require a multi-factorial approach as utilized in HAART, a multi-factorial approach may be necessary to target multiple host cell proteins and/or multiple HIV proteins to ensure long-term benefit without the possibility of generating escape mutants. Another possible approach is to identify the most highly conserved regions of the HIV transcript, specifically the intron/exon junctions, and generate siRNAs that target these regions. A combination of all these novel approaches will be necessary to combat the virus and if HIV overcomes the siRNAs to known cellular proteins (NFκB, HIV receptor CD4, the co-receptors CCR5 and CXCR4, LEDGF/p75, BST-2, and others), new cellular proteins required for HIV infection will need to be identified.

Uses of retroviral vectors: small scale functional genomics

Given that retroviral vectors are capable of inducing long-term stable gene expression, one of its first applications was expression cloning. This system was first used to identify the cellular co-receptors necessary for SIV infections (Deng et al., 1997). These co-receptors were identified by first generating an MLV based human T-cell cDNA library. This MLV cDNA library was then used to infect 3T3.CD4 cells (these cells are unable to be infected by virus pseudotyped with either the $SIV_{agmTY01}$ or $SIV_{mac1A11}$ Env glycoproteins). The transduced 3T3.CD4 cells were then challenged with a lentiviral vector encoding the gene for puromycin resistance and pseudotyped with

either the $SIV_{agmTY01}$ or $SIV_{mac1A11}$ Env glycoproteins. Cells that were capable of being infected at this point now possessed a selectable marker. After selection with puromycin, cells that were now susceptible to transduction harbored the co-receptor required for infection through the $SIV_{agmTY01}$ or $SIV_{mac1A11}$ Env glycoproteins, and this cDNA could be identified using PCR. Another area that benefited from the use of expression cloning with MLV retroviral vectors was the identification of players in the TNF and Fas mediated apoptosis pathways. Here researchers generated MLV retroviral cDNA libraries from cell lines that were resistant to apoptosis, infected cells that were susceptible to apoptosis, challenged the susceptible cells with TNF or Fas, and isolated cDNA from the population of cells that become resistant. Using this method, the transcriptional activator BSAC was shown to induce resistance to TNF mediated apoptosis (Sasazuki et al., 2002) and adhesion protein ICAM-2 (Perez et al., 2002) and membrane protein Lifeguard (Somia et al., 1999) were shown to induce resistance to Fas mediated apoptosis.

Retroviral cDNA libraries have more recently been used to identify proteins that inhibit HIV infection. An MLV retroviral cDNA expression library was used to isolate *Lv1*, a restriction factor responsible for HIV resistance in old world monkeys (Stremlau et al., 2004). This restriction factor, Trim5α, was cloned by generating an MLV retroviral cDNA library from rhesus monkeys resistant to HIV-1 vector infection and infecting HeLa cells that are permissive to HIV-1 vector infection. These HeLa cells were than infected with a lentiviral vector encoding EGFP and FACS sorted through multiple rounds of infection to find populations of cells that were completely resistant to infection (i.e. GFP negative). The cDNA was isolated from these EGFP negative populations and

the restriction factor was identified. In other studies, MLV cDNA expression libraries were used to identify genes that conferred resistance to retrovirally mediated transduction in human cell lines. The N-terminal fragment of the heterogeneous nuclear ribonuclear protein U (hnRNP) was shown to inhibit HIV gene expression (Valente and Goff, 2006) and a CCCH-type zinc finger protein designated ZAP was shown to specifically inhibit the accumulation of retroviral RNA in the cytoplasm (Gao et al., 2002). The use of MLV retroviral cDNA libraries for expression cloning gave researchers another tool to identify novel genes and correlate them with a specific function.

Given that lentiviral vectors have a nearly identical life cycle to HIV (Fig 1-3 vs. Fig 1-6), it is not surprising that these vectors have been used to recapitulate the early phase of HIV infections and identify host cell factors that are necessary for HIV infection or suppress HIV infection in the early phases. Several studies, including those that will be described in subsequent chapters of this thesis, have mutagenized cell lines and isolated clonal populations of cells that are resistant to retrovirally mediated transduction. In one study, researchers isolated populations of cells that were resistant to MLV based retroviral vectors but completely susceptible to lentiviral vectors (Bruce et al., 2005). Later these individuals implicated the host cell sulfonation pathway in their resistant line (Bruce et al., 2008). This resistance phenotype was specific to LTR mediated gene expression. Consequently, the cell line was resistant to MLV based retroviral vectors that possess intact LTRs, but susceptible to HIV based lentiviral vectors that possess incomplete LTRs. In another study, Rat2 cells were mutagenized and challenged with a MLV retroviral vector encoding HSV-*TK* (Gao and Goff, 1999). The mutagenized populations were then challenged with trifluorothymidine (TFT) which killed the HSV-

TK positive cells. Two clonal populations were isolated that were resistant to both lentiviral and MLV retrovirally mediated transduction. One clone was resistant at a stage after reverse transcription and another was resistant at a stage prior to reverse transcription. Ultimately these researchers showed that the resistance phenotype in one cell line was the result of overexpression of the fasciculation and elongation protein ζ-1 (FEZ1) (Naghavi et al., 2005). These studies have shown that the use of retroviral vectors as a means for understanding HIV is appropriate given that lentiviral vectors recapitulate the early life cycle of HIV and utilize the same intracellular host machinery.

Uses of retroviral vectors: large-scale functional genomics

Two recent large scale functional genomic studies have attempted to identify all the genes that play a role in both early HIV infectious events (binding, uncoating, import, integration, and expression) and in late infectious events (transcription, mRNA processing, translation, assembly, and budding). A third large scale screen only looked at the early HIV infections events. For a comparison of the study conditions see Table 1-3. The first screen used HeLa cells that expressed CD4 and a β-galactosidase reporter gene responsive to Tat (Brass et al., 2008). For each gene, pools of four siRNAs were transfected into the HeLa cells seventy-two hours before infection with a replication competent virus. Forty-eight hours after infection the supernatant from the cells was set aside and the cells were analyzed for the presence of the HIV capsid protein p24. The reserved supernatants, which contained any released virus, were then used to infect new HeLa cells and late infectious events were monitored using the β-galactosidase reporter. The second screen used 293T cells and a luciferase reporter gene (Konig et al., 2008).

Table 1-3: Comparison of Three 2008 Large Scale Function Genomic Studies to identify HIV interactors

Study	Cell Line Used	Virus Used	Length of siRNA Treatment	Post Infection Readout Timepoints	Assay System	# of Hits
Brass et. al, 2008	HeLa (CD4+; β-gal reporter)	replication competent	72 hours	early infection- 48 hours; late infection- 48 hours	early infection- p24; late infection- β-gal reporter	273
Konig et. al, 2008	293T	lentiviral vector	48 hours	early infection only- 24 hours	luciferase	295
Zhou et. al, 2008	HeLa (CD4+; β-gal reporter)	replication competent	24 hours	48 hours; 96 hours	β-gal reporter	224

Table 1-3

Comparison of siRNA studies.

Comparison of conditions and results of three large scale functional genomics studies to identify host cell proteins necessary for HIV. Although these three studies were attempting to identify the same proteins there was very little overlap. This is likely due to the variable conditions utilized by each study. β-Gal- Beta galactosidase.

For each gene, pools of approximately six siRNAs were transfected into the 293T cells forty-eight hours before infection with a lentiviral vector pseudotyped with VSV-G and encoding the reporter luciferase. By using a lentiviral vector and VSV-G this screen only looked at events post entry and before the late infectious events. The third study was very similar to the study by Brass and colleagues in that the screen was also performed in HeLa cells that expressed CD4 and a β-galactosidase reporter gene responsive to Tat (Zhou et al., 2008). For each gene, pools of approximately three siRNAs were transfected into the HeLa cells twenty-four hours before infection with a replication competent virus. This screen was unique in that it examined β-galactosidase activity at two timepoints: 48 hours post infection to detect virus entry and 96 hours post infection to detect virus spreading into additional cells.

When the results from these three screens were compared, it was quite surprising that there was very little overlap between the genes identified. Of all the genes identified, only three genes were common to all three screens (MED6, MED7, and RELA) (Bushman et al., 2009). The difference in sets of genes identified can be accounted for by different assay systems, different viruses utilized in the studies, experimental noise, timing of sampling, and different filtering criteria (Bushman et al., 2009). What these studies show is that siRNA is a powerful tool that can be used to identify a wide range of host cell proteins, some of which can become potential drug targets (Bushman et al., 2009). These new drugs will likely be more difficult for HIV to become resistant to since they are host cell proteins and not HIV proteins. The search for host cell factors is on going and eventually a host cell factor will be found that satisfies all of the following

criteria: it is critical for HIV infection, it is an effective druggable target, and will reduce HIV to merely a chronic and non-lethal condition.

CHAPTER 2

A barnase based screen to isolate cell populations resistant to retroviral infection

Introduction

Retroviruses have been associated with many debilitating mammalian diseases including human T-cell leukemia virus (HTLV) (Gallo, 1981) and human immunodeficiency virus (HIV) (Barre-Sinoussi et al., 1983), but recently they have been harnessed to aid human health rather than harm it. Retroviral vectors derived from retroviruses including Moloney Murine Leukemia Virus (MLV) and HIV are being used in gene therapy applications to deliver long term expression of specific transgenes while attempting to minimize any adverse side effects (Sinn et al., 2005). The use of toxic gene products as the transgene in viral vectors has been examined extensively for therapies involving HIV infection (Caruso and Klatzmann, 1992; Harrison et al., 1991), the treatment of cancer (Ram et al., 1993), and for positive/negative *ex vivo* selection (Couderc et al., 1999). From these studies and many others (reviewed in (Portsmouth et al., 2007)), two questions present themselves: what toxic gene should be used and how to regulate its expression.

Several toxic gene products have been utilized in viral vectors including cytosine deaminase (Mullen et al., 1992) and herpes simplex 1 virus thymidine kinase (HSV-*TK*) (Moolten, 1986). In the latter system, the *TK* converts the prodrug gancyclovir into a nucleotide analogue that competitively inhibits the incorporation of dGTP into DNA thereby preventing DNA elongation and causing cell death (Frank et al., 1984). Thus, cellular toxicity is controlled by the administration of the prodrug. Although this seems advantageous since the gene product itself is not toxic, the administration of gancyclovir

in vivo is not 100% effective and cells containing HSV-*TK* may be resistant to the prodrug (Beck et al., 1995; Carrio et al., 2001; Sturtz et al., 1997). One potential advantage to this system has been observed as a "bystander" effect (Bi et al., 1993). This is when untransduced neighboring cells are capable of taking up HSV-*TK*, either *in vivo* or *in vitro*, from a transduced neighbor and converting ganyclovir into the lethal product. However, the pitfall of this situation is that untargeted cells can undergo cell death at an inopportune time. HSV-*TK* is the preferred system for toxic gene therapy and even with its limitations it is still being used extensively in cancer suicide gene therapy (Ahn et al., 2009; Rath et al., 2009; Saito et al., 2009; Song et al., 2009).

One problem with suicide gene therapy is that the suicide gene is constitutively active and will be expressed even if it is not being used. The mechanism to alleviate this problem, and bring a level of specificity to suicide gene therapy, is to use inducible or tissue specific promoters. Inducible promoters can be created with a minimal basal promoter along with the bacterial Tet system (Gossen and Bujard, 1992). This synthetic promoter can be induced or repressed by a prodrug like doxycycline, which inhibits the binding of an operator to the DNA. Gene therapy is also using tissue specific promoters to limit the expression of a gene of interest to a specific cell type. Promoters have been identified or synthesized that limit expression to specific tissues including muscle (Fassati et al., 1998), liver (Peng et al., 1988), or the central nervous system (Cortez et al., 2000). Additionally, the PSA positive regulatory sequence (PSAR), a promoter that limits expression to the prostate specific antigen (PSA) positive epithelial cells of the prostate (Pang et al., 1995), has been used to specifically kill prostate cancer cells. It has been demonstrated that prostate cancer development and progression correlates with

elevated PSA levels (Stamey et al., 1987). In order to selectively kill the PSA positive epithelial cells that lead to prostate cancer, the PSAR has been used to generate lentiviral vectors that express a suicide gene. Consequently this lentiviral vector kills PSA positive prostate cells while not killing the wildtype cells within the same culture (Yu et al., 2001). The use of tissue specific promoters in conjunction with a retroviral vector for gene expression opens up the possibility of utilizing the same strategy for other cancers and diseases.

Previous work has validated the use of toxic retroviral constructs to study and identify novel proteins in the HIV lifecycle through loss of function analysis (Gao and Goff, 1999). Rat-2 cells deficient for thymidine kinase were repeatedly mutagenized and subsequently challenged with a MLV vector encoding HSV-*TK*. In theory, cells that were successfully transduced were killed in the presence of the prodrug trifluorothymidine (TFT). TFT is converted to a thymidine analog by the HSV-*TK* gene product and causes repression of DNA synthesis, leaving surviving cells that were resistant to infection. The authors successfully isolated two clones of Rat-2 cells refractory to infection by ten to one hundred fold as compared to the parental Rat-2 cell line. Further analysis utilizing Affymetrix gene chips comparing the expression pattern of the parental Rat-2 cell line to one of the mutant clones, identified fasciculation and elongation protein ζ-1 (FEZ1). FEZ1 was upregulated approximately 30 fold in the resistant cell line and when these elevated levels of FEZ1 were knocked down to wildtype levels using small interfering RNA the resistance phenotype disappeared (Naghavi et al., 2005). These studies showed it was possible to first isolate clonal

populations of cells resistant to retrovirally mediated transduction and that it was possible to identify the gene that conferred the resistance phenotype.

The major limitation in creating non-prodrug based toxic retroviral vectors has been an inability to eliminate the toxic effects in the packaging cell line. During the production phase of the toxic retroviral vector, the toxic gene product is transcribed and translated in the packaging cell, causing death of the packaging cell line. An ideal system would be one in which the toxic activity is repressed in the packaging cell line and only fully activated upon a productive infection. Here we report a novel method of generating a toxic retroviral vector based on the previously characterized toxic gene product, barnase. Barnase is an extracellular ribonuclease derived from *Bacillus amyloliquefaciens* (Hartley, 1988) and has been shown to be toxic to cells in culture (Leuchtenberger et al., 2001). *Bacillus amyloliquefaciens* expresses an inhibitor barstar that inhibits barnase by binding to it stoichiometrically (Hartley, 1988). The existence of a potent inhibitor allows for the creation of a high titer toxic retroviral vector that does not require an additional prodrug. By repressing the toxic properties within the packaging cell line with the inhibitor barstar, the packaging cell line and consequently virus generation are protected. Only after the barnase virus successfully infects a target cell that does not contain the inhibitor barstar will the toxic properties of barnase be apparent and induce cell death.

We constructed two distinct retroviral vectors that transduce the barnase gene and upon productive infection induce cell death. These two retroviral vectors were based on MLV and HIV retroviruses, allowing us to make general conclusions about the utility of this system with retroviral vectors. The cell death caused by these retroviral vectors is

evident in cells derived from a wide variety of tissues, across various species, and includes both cancerous and non-cancerous cell lines. We also show that barstar is critical to generate high titer toxic retroviral vectors, since the absence of it leads to a lower titered virus. Finally, we show an application of this novel toxic retroviral vector in the isolation of a cell line refractory to retroviral infection. We propose that by using inducible and/or tissue specific promoters, along with specific cell targeting by retroviruses, this novel mechanism of cell killing may have applications in the fields of cancer biology, targeted cell destruction, and as a mechanism to elucidate the HIV lifecycle through loss of function genetics.

Results

Vector Generation

In order to generate a cell line resistant to retroviruses we first developed toxic retroviral vectors based on barnase. Retroviral vectors are synthetic retroviruses that recapitulate a portion of the life cycle of the retrovirus from which they are derived. Both lenti-barnase and MLV-barnase were developed and compared to determine their respective killing efficiencies. Additionally, in order to determine whether or not the inhibitor barstar was essential to generate high titer virus, a barstar expression plasmid was created to inhibit the toxic barnase activity in the packaging cell line. Figure 2-1 illustrates the constructs used for this study.

The lentiviral vectors used in this study were generated from the CS family of self inactivating lentiviral vectors (Miyoshi et al., 1998). These third generation lentiviral vectors contain deletions of all non-essential HIV proteins and only contain: a CMV/HIV

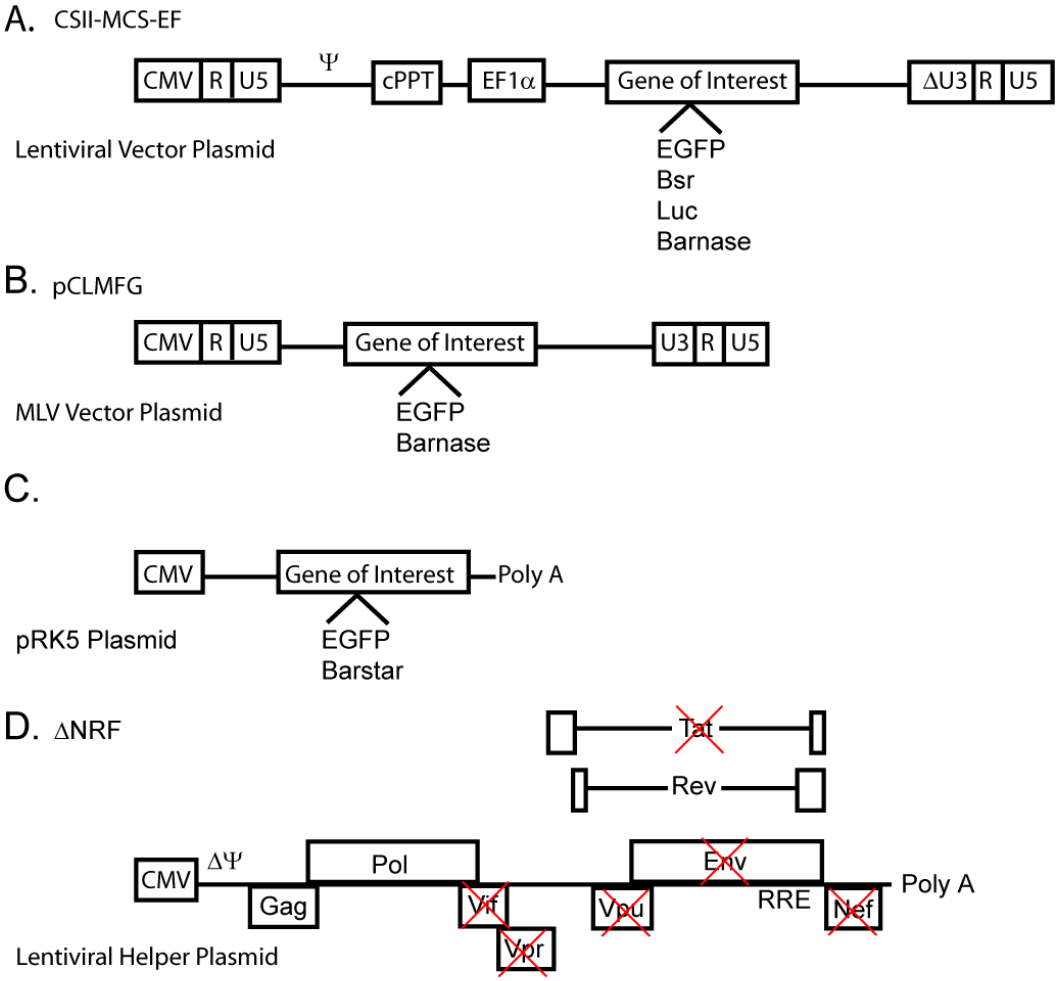

A. CSII-MCS-EF
CMV
R
U5
Ψ
cPPT
EF1α
Gene of Interest
ΔU3
R
U5
Lentiviral Vector Plasmid
EGFP
Bsr
Luc
Barnase
B. pCLMFG
CMV
R
U5
Gene of Interest
U3
R
U5
MLV Vector Plasmid
EGFP
Barnase
C.
CMV
Gene of Interest
Poly A
pRK5 Plasmid
EGFP
Barstar
D. ΔNRF
Tat
Rev
CMV
ΔΨ
Gag
Pol
Vif
Vpr
Vpu
Env
RRE
Nef
Poly A
Lentiviral Helper Plasmid
E.
CMV
Envelope
Poly A
Envelope Plasmid
VSVG
Ampho

Figure 2-1

Constructs used in this study.

(A) CSII-EF-MCS is a self inactivating lentiviral vector plasmid that contains the packaging sequence (Ψ), lacks all non-essential HIV proteins, contains a replacement of the 5' U3 with the CMV promoter and harbors a partial deletion in the 3' U3 region of the LTR (ΔU3). The promoter, derived from human elongation factor 1α (EF1α), was used to regulate expression of a gene of interest. Genes for enhanced green fluorescent protein (EGFP), blasticidin resistance (Bsr), luciferase (Luc) and barnase were cloned into CSII-EF-MCS. (B) pCLMFG Moloney retroviral vector plasmids were used to generate MLV-based viral vectors. This vector was used to transduce EGFP or barnase. (C) pRK5 constructs are used to transcriptionally regulate expression of either EGFP or barnase. Barstar was expressed to inhibit barnase function in the packaging cell line, and EGFP was used to monitor transfection efficiency when generating virus. (D) ΔNRF is a helper plasmid that contains all the structural and enzymatic proteins necessary to create a lentiviral vector while at the same time harboring deletions of all non-essential HIV accessory proteins. It is necessary to co-transfect ΔNRF along with an envelope protein to create a lentiviral vector. (E) Two envelope proteins were utilized in this study. VSV-G was derived from the Vesicular Stomatits Virus G-protein and Ampho is the 10A1 amphotrophic envelope. Ampho- amphotrophic 10A1 envelope; Bsr- blasticidin resistance; cPPT- central polypurine tract CMV- cytomegalovirus virus; EF1α- elongation factor 1α; EGFP- enhanced green fluorescent protein; Luc- luciferace; RRE- rev response element; VSVG- vesicular stomatitis virus g-protein.

LTR hybrid promoter followed by the packaging signal (Ψ), a gene of interest expression cassette consisting of the internal promoter and the gene of interest, and the 3' self-inactivating (SIN) LTR (Fig 2-1A). The CS lentiviral vector used in this study utilized the human elongation factor 1α promoter (EF1α) to regulate expression of the gene of interest in the target cell. This promoter has been shown to fuction in a wide host range and allows for high levels of gene expression (Kim et al., 1990). Furthermore, the lentiviral vector contains the central polypurine tract (cPPT), a central DNA flap that acts as a cis-determinant of HIV-1 DNA nuclear import and facilitates integration of the viral genome into non-dividing cells (Zennou et al., 2000). Lenti-barnase was created to determine how efficiently lentiviral vectors could kill a target cell. Lenti-EGFP, lenti-bsr, and lenti-luciferase were all used to further characterize the resistant cell line. All necessary HIV proteins, specifically *Gag*, *Pol*, *Rev*, and *Env*, are provided *in trans* via ΔNRF and the envelope plasmid (Fig. 2-1D and Fig. 2-1E). HIV retroviral vectors were generated by transient transfection of a human embryonic kidney cell line 293T (see chapter 5 for Materials and Methods).

The MLV vectors used in this study are based on the vectors designed by Dranoff (Dranoff et al., 1993) that have undergone a slight modification to generate the pCL system (Naviaux et al., 1996). The notable difference between the original Dranoff vector and the pCLMFG vector utilized in this study has been the replacement of the 5' U3 region with the immediate early promoter of the cytomegalovirus virus (CMV) promoter leading to increased viral titers. This increase in viral titer occurs because the expression of the retroviral RNA is controlled in the packaging cell line by the CMV promoter and the expression of RNA in the target cells is regulated by the LTR sequences

(Naviaux et al., 1996). MLV-barnase was generated to determine how efficiently MLV-based retroviral vectors could kill target cells and MLV-EGFP was used to further characterize the resistant clone (Fig. 2-1B). In order to complete the generation of MLV virus in the packaging cell line, additional helper plasmids were provided *in trans* (an envelope protein (Fig. 2-1E) and other viral proteins (CMV-Gag-Pol, not shown)).

The expression construct pRK5 (BD Biosciences) is a mammalian expression construct that expresses a gene of interest using the CMV early promoter. I generated pRK5-barstar to express the inhibitor of barnase (barstar) in the packaging cell line during production of viral vectors encoding barnase (Fig. 2-1C).

Virus Generation

Viral vectors transducing barnase were generated by co-transfecting 293T cells with the viral vector plasmid (lenti-barnase or MLV-barnase) and the respective helper plasmids with or without the expression plasmid for the inhibitor, pRK5-barstar. Both MLV-barnase and lenti-barnase were generated and tested in order to determine the difference in killing efficiencies between virus types and cells types targeted. Additionally, to monitor the efficiency of transfection, pRK5-EGFP was also co-transfected. Using fluorescence microscopy, I examined EGFP expression in 293T cells that were transfected to produce barnase virus with or without the presence of the barnase inhibitor barstar (Figure 2-2). When barstar was present, EGFP expression was greatly enhanced (Fig. 2-2A) and the absence of inhibitor lead to greatly reduced levels of EGFP (Fig. 2-2B). We postulate that in the absence of barstar all transcripts, including the transcript encoding EGFP and/or transcripts that encode proteins required for EGFP expression, are degraded by barnase due to its nonspecific RNase activity.

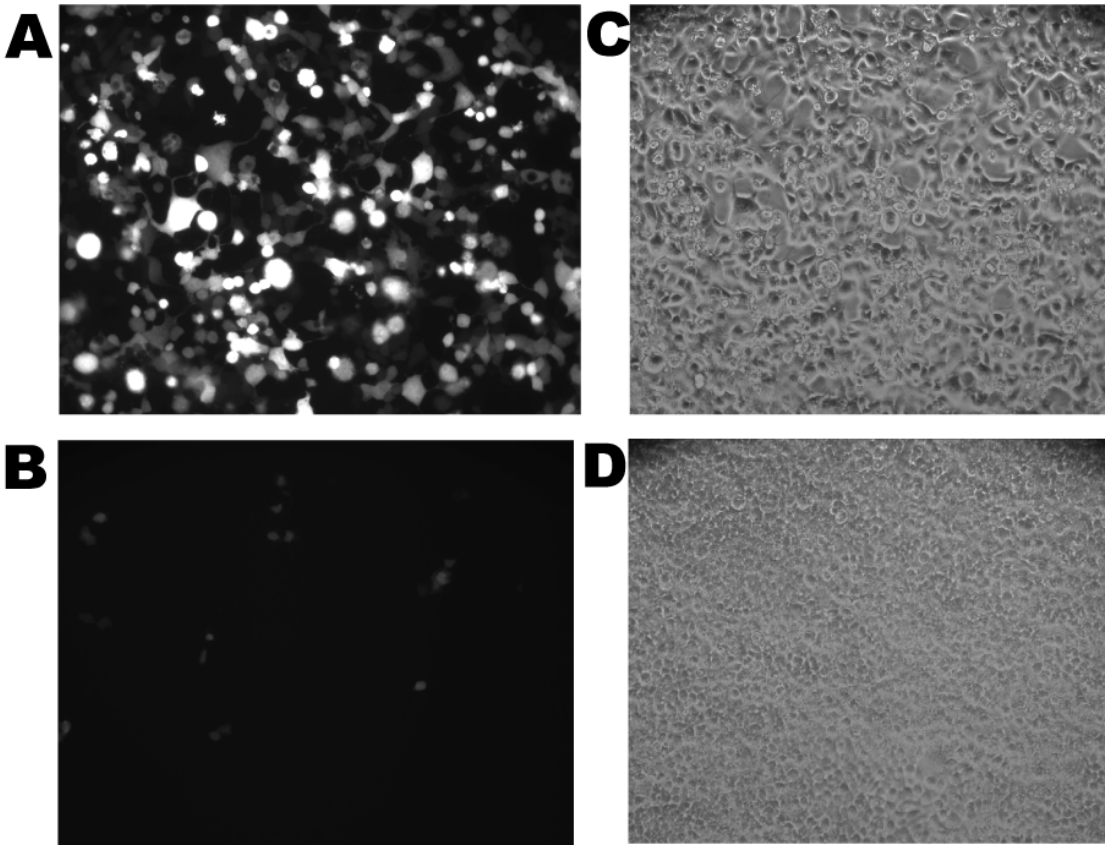

A
C
B
D

Figure 2-2

GFP expression is greatly enhanced when MLV-barnase is generated in the presence of barstar.

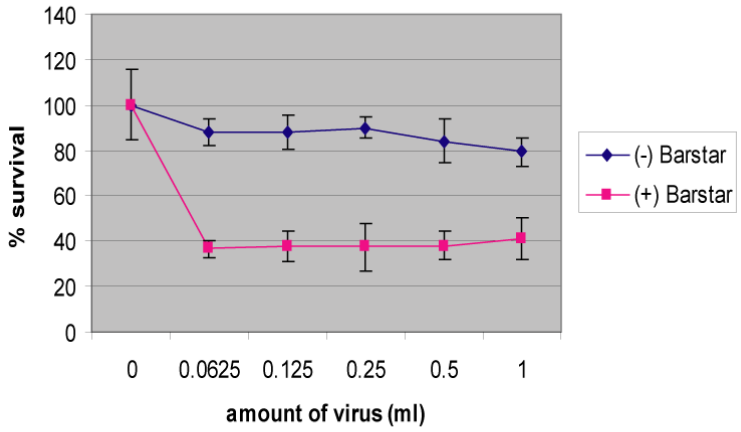
MLV-barnase was generated in 293T cells in the presence of the barnase inhibitor barstar (A, fluorescence; C, brightfield) or in the absence of the inhibitor (B, fluorescence; D, brightfield). An EGFP expression vector was cotransfected to monitor transfection efficiency. The exposure time of the image in A was 400 ms while the exposure time of the image in B was extended to 800 ms to visualize the low level of GFP expression. Similar results were observed when lenti-barnase was generated in the presence or absence of barstar.

Quantification of Barnase Mediated Cell Death

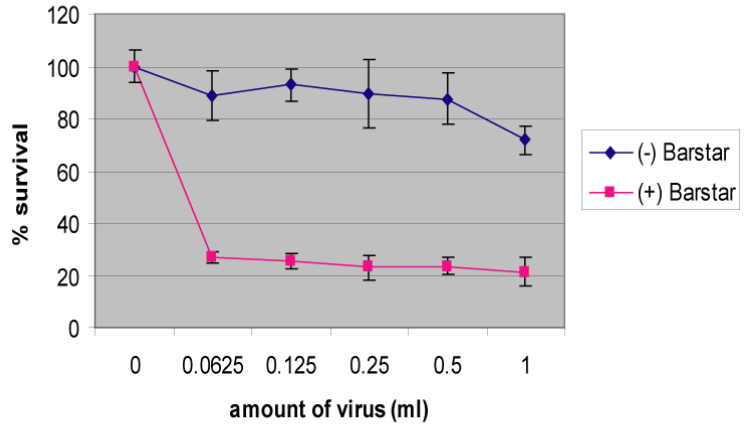
To determine the efficacy of killing, we tested the ability of the viral vectors transducing barnase to kill cell lines from multiple species. Human (HeLa, DU-145, and A-498) and hamster (V79-4) cell lines were infected with varying amounts of viral vector and cell survival was measured 48 hours later. To minimize titer differences between batches of virus, single large productions of both MLV- and lentiviral-based barnase virus were generated and applied to four distinct cell lines. I observe that both lentiviral- and MLV-based barnase viruses were able to infect and kill both hamster and human cell lines. The results indicated that barnase mediated killing occurs in a dose dependent manner with some variable killing efficiency between cell lines (Fig. 2-3). This variability is likely a result of different cell surface proteins found on kidney, prostate, lung or cervical cancer derived cell lines or other host cell factors that may play a role in susceptibility to barnase based toxicity and/or vector infection.

Virus generated in the presence of the inhibitor barstar (Fig. 2-3; squares) demonstrated a markedly greater level of toxicity to target cells compared to virus generated in the absence of barstar (Fig. 2-3; diamonds). Greater toxicity to target cells probably resulted from higher viral titers obtained when the packaging cell lines also contained the inhibitor barstar. We postulate that the inhibitor prevents barnase-based toxicity to the packaging cell line leading to higher yields of virus. Although viral titers were reduced, productive virus is not completely absent from the inhibitor deficient packaging cell lines. One possible explanation for this observation is that packageable viral RNA is the rate-limiting step in generating productive retroviral vectors (Naviaux et al., 1996). If the viral RNA that encodes for barnase is rapidly packaged into virions,

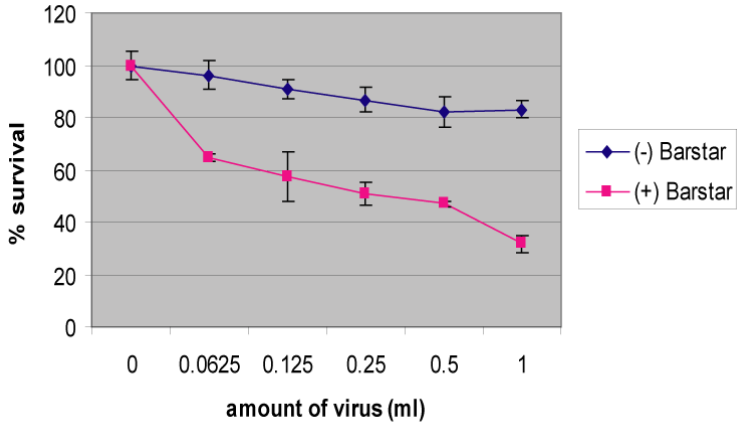
A(1)


MLV-Barnase Killing of DU-145
% survival
140
120
100
80
60
40
20
0
0
0.0625
0.125
0.25
0.5
1
amount of virus (ml)
(-) Barstar
(+) Barstar

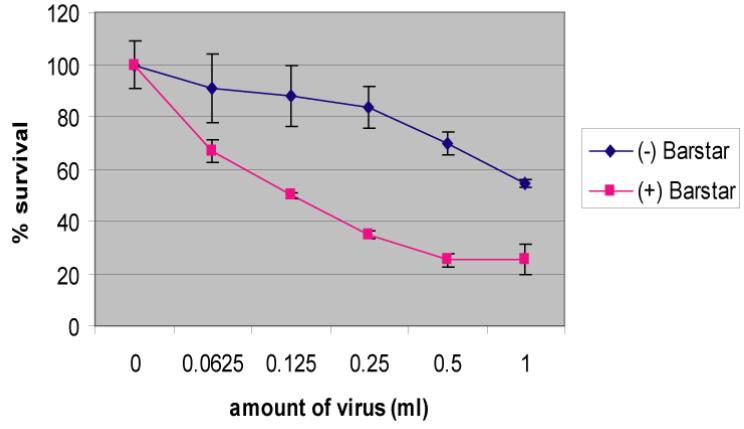
A(2)


MLV-Barnase Killing of HeLa
% survival
120
100
80
60
40
20
0
0
0.0625
0.125
0.25
0.5
1
amount of virus (ml)
(-) Barstar
(+) Barstar

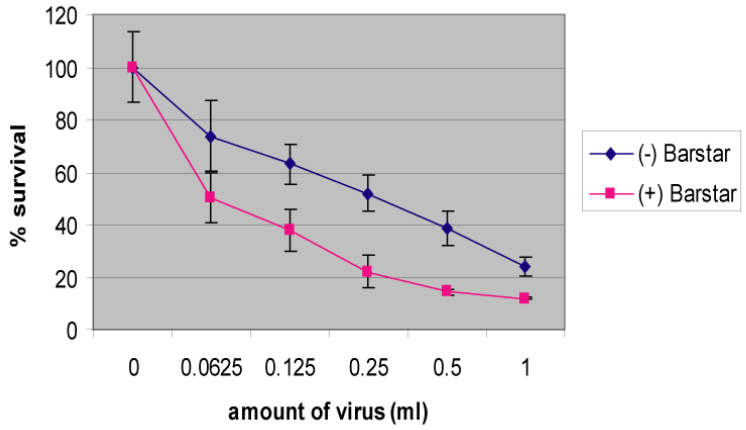
A(3)


MLV-Barnase Killing of A-498
% survival
120
100
80
60
40
20
0
0
0.0625
0.125
0.25
0.5
1
amount of virus (ml)
(-) Barstar
(+) Barstar

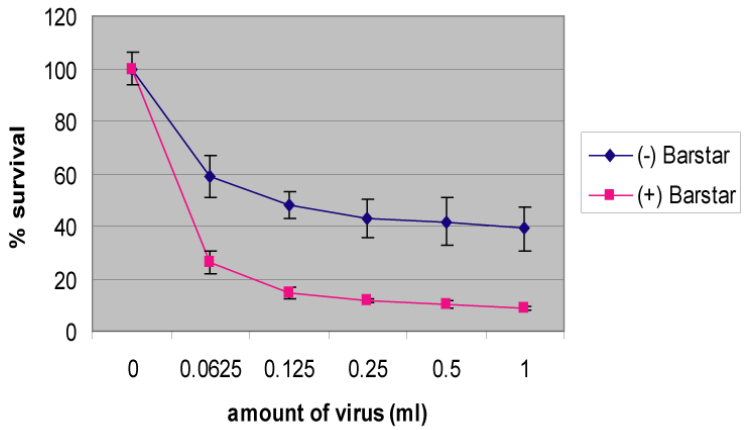
A(4)


MLV-Barnase Killing of V79-4
% survival
120
100
80
60
40
20
0
0
0.0625
0.125
0.25
0.5
1
amount of virus (ml)
(-) Barstar
(+) Barstar

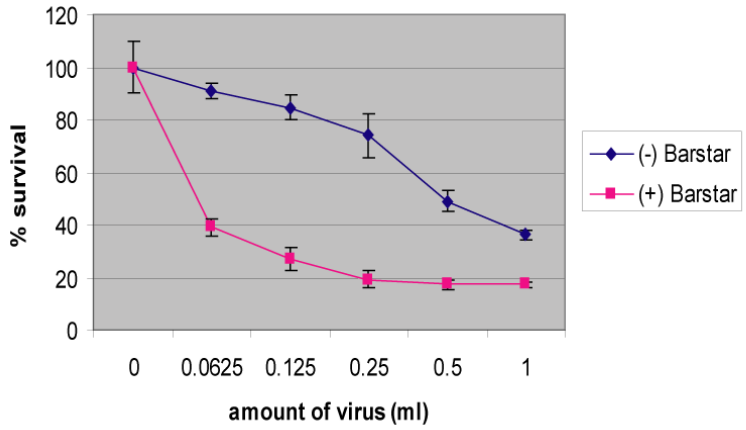
B(1)


Lenti-Barnase Killing of DU-145
% survival
120
100
80
60
40
20
0
0
0.0625
0.125
0.25
0.5
1
amount of virus (ml)
(-) Barstar
(+) Barstar

B(2)


Lenti-Barnase Killing of HeLa
% survival
120
100
80
60
40
20
0
0
0.0625
0.125
0.25
0.5
1
amount of virus (ml)
(-) Barstar
(+) Barstar

B(3)


Lenti-Barnase Killing of A-498
% survival
120
100
80
60
40
20
0
0
0.0625
0.125
0.25
0.5
1
amount of virus (ml)
(-) Barstar
(+) Barstar

B(4)

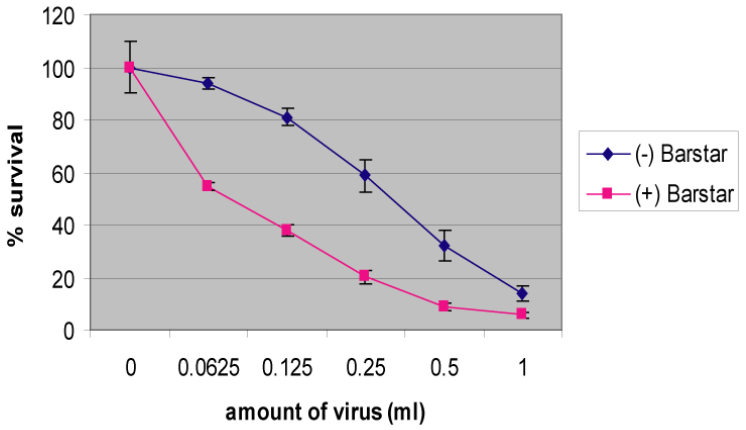

Lenti-Barnase Killing of V79-4
% survival
120
100
80
60
40
20
0
0
0.0625
0.125
0.25
0.5
1
amount of virus (ml)
(-) Barstar
(+) Barstar

Figure 2-3

Generation of barnase vector in the presence of barstar yields higher titer virus. (A-1) MLV-barnase mediated killing of DU-145 (human prostate cancer line), (A-2) MLV-barnase killing of HeLa (human cervical carcinoma line), (A-3) MLV-barnase killing of A-498 (kidney carcinoma line), (A-4) MLV-barnase killing of V79-4 cells (hamster lung fibroblast line). (B-1) Lenti-barnase killing of DU-145, (B-2) Lenti-barnase killing of HeLa, (B-3) Lenti-barnase killing of A-498, (B-4) Lenti-barnase killing of V79-4. For both (A) and (B), cells were infected with two fold dilutions of either barnase retrovirus generated with barstar (squares) or barnase retrovirus generated without barstar (diamonds). Results were obtained in quadruplicate for each point, the mean calculated, and plotted to indicate the percent survival at each dose compared to uninfected cells. More efficient cell killing occurs with vector generated in the presence of barstar (comparing percent survival at identical dose amounts) indicating higher titers of virus are generated in the presence of barstar.

protein expression may not occur in the packaging cell line and therefore some productive retroviral vectors will be produced even in the absence of the inhibitor barstar.

Additionally, variability was observed between MLV- (Fig. 2-3A) and lentiviral-based (Fig. 2-3B) barnase vectors. Two trends were observed when comparing the infections of these two different viruses. First, at nearly every point, when comparing the same cell lines and same viral titers, there was decreased survival when cells were infected by the lentiviral vector compared to cells infected by the MLV vector. This observation may be explained by the previous findings that MLV vectors can only infect cells that are undergoing mitosis (Roe et al., 1993) and that the higher percentage of surviving cells in MLV vector infections occurred because not all cells had not undergone mitosis. Second, lentiviral-based barnase vectors have an overall higher level of saturated killing as compared to MLV-based barnase vectors. This observation may be a result of the mechanism used to regulate the expression of barnase. It is possible that the EF1α promoter in the lentiviral vector is more efficient and stronger than the LTRs found in the MLV vector. Additionally, since lentiviral vectors are capable of infecting both dividing and non-dividing cells (Naldini et al., 1996), there is a greater opportunity for a productive infection, and therefore a higher rate of killing by lentiviruses compared to killing rates due to MLV infection.

Generating a Retroviral Resistant Clone

We selected V79-4 cells for this study. V79-4 cells are male Chinese hamster lung fibroblasts that were shown to be amenable to mutagenesis studies (Lee et al., 1995; Thacker, 1981; Thacker et al., 1994). Additionally, karyotype analysis has revealed that V79-4 cells have lost approximately 14% of their chromosome arm length while

maintaining at least one copy of each autosome and only one X chromosome (Thacker, 1981). This effectively makes V79-4 up to 28% haploid and generation of loss of function somatic mutants is facilitated since this event requires one hit kinetics. Notably, the hypoxanthine guanine phosphoribosyltransferase (HPRT) gene is located on the X chromosome and is haploid in the male V79-4 cells and the adenine phosphoribosyltransferase (APRT) is present on autosomal chromosome 9 and diploid in V79-4 cells (Colella et al., 1988). Since V79-4 is haploid for HPRT and functionally diploid for APRT and there are powerful selection strategies to select against the expression of these enzymes, we used these loci to assess the efficacy of our mutagenesis procedure.

To induce mutations, V79-4 cells were challenged with ICR-191, an acridine agent capable of inducing frameshift mutations, small deletions, and chromosomal rearrangements (Ferguson and MacPhee, 1983). Figure 2-4 illustrates the protocol used to mutagenize the cells. Our goal using this approach was to generate a cell line with many null mutations in many different genes. Mutations in essential genes would kill the cell, but mutations in non-essential genes would permit survival. Ideally, we were looking to identify null mutants in genes essential for retrovirally mediated transduction and not essential for survival. Ten plates with 1 x 10^6 V79-4 cells were challenged with ICR-191 for 10 hours and allowed to recover post mutagenesis until the plates were confluent. The plates were pooled to generate heterogeneous populations and analyzed for the presence of HPRT and APRT null cells. 1 x 10^6 post mutagenesis cells were selected with 6-thioguanine (6-TG) to identify HPRT null cells or diaminopurine (DAP) to identify APRT null cells. Both HPRT and APRT are involved in the purine salvage

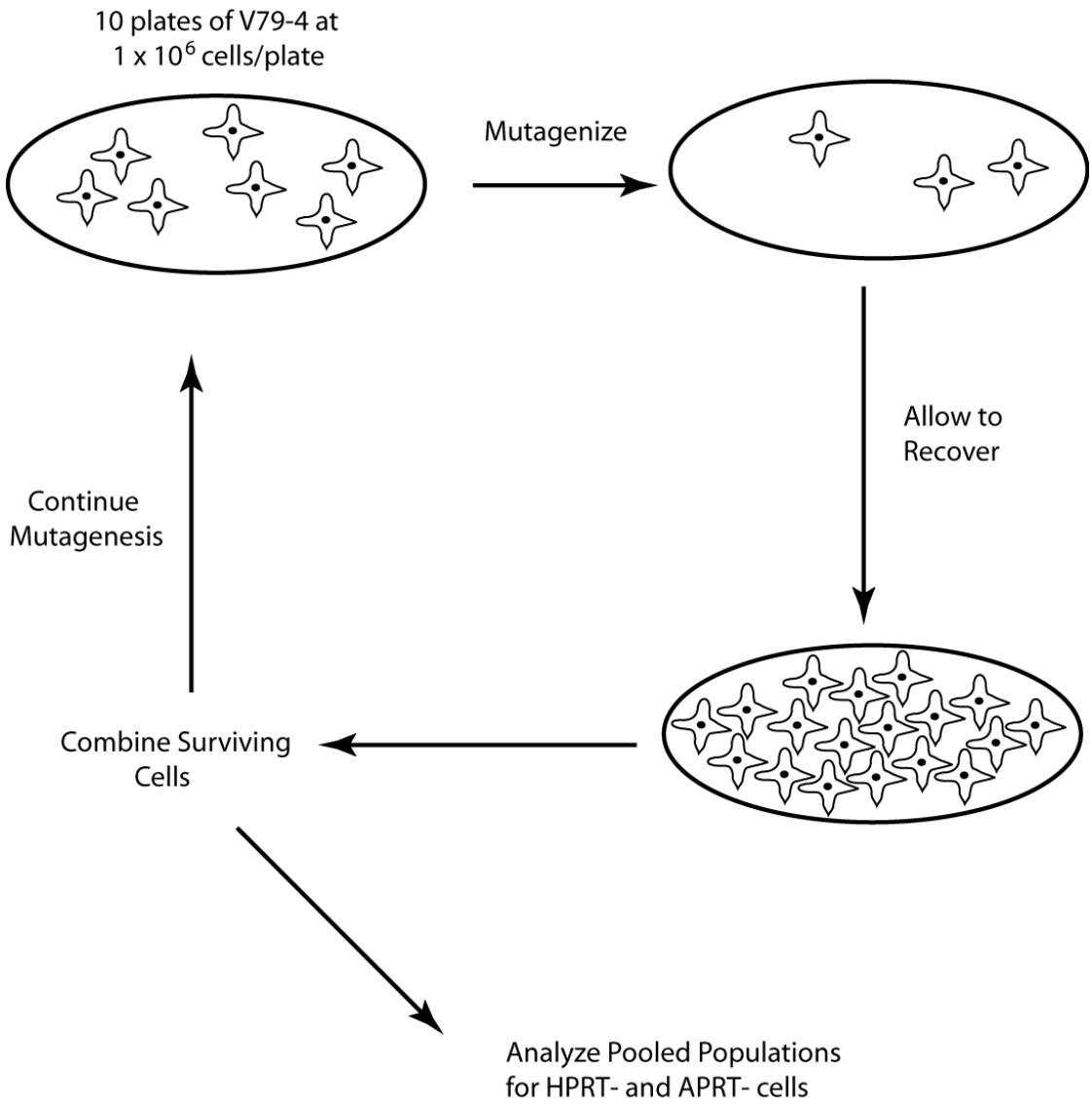

10 plates of V79-4 at
1 x 10^6 cells/plate
Mutagenize
Allow to
Recover
Continue
Mutagenesis
Combine Surviving
Cells
Analyze Pooled Populations
for HPRT- and APRT- cells

Fig. 2-4

Mutagenesis protocol.

Ten plates of V79-4 cells at a density of 1 x 10^6 cells were mutagenized with ICR-191 and allowed to recover to confluency. The surviving cells of all ten plates were then pooled and either subjected to further mutagenesis or analyzed for the presence of HPRT or APRT deficient populations. Cells after the fourth round of mutagenesis were used for further analysis. APRT- adenine phosphoribosyltransferase; HPRT- hypoxanthine phosphoribosyltransferase.

pathway. When cells are HPRT or APRT null and in the presence of either 6-TG or DAP, they will convert either 6-TG or DAP to a toxic nucleotide. This nucleotide will then incorporate into the DNA of the cell, leading to cell death. The appearance of APRT null cells was greatly reduced compared to HPRT null cells (Table 2-1). This is expected due to the number of copies of each gene present in V79-4 cells. While 6-TG resistant cells (HPRT null colonies) were observed at a high frequency after a single round of mutagenesis (1 in 10^5 cells), we only isolated a large number of APRT deficient clones after three rounds of mutagenesis. Additionally, the occurrence of APRT deficiency was at a lower rate than that of HPRT deficiency, with 22 clones isolated from 10^7 cells after the fourth round of mutagenesis. These results demonstrate that our mutagenesis protocol can effectively alter expression or activity of alleles of haploid as well as diploid genes. Additionally, altered expression of a diploid gene can be isolated in a large population of the cells (greater than 2 in 10^6) only after several rounds of mutagenesis.

Once we determined that our mutagenesis protocol was capable of isolating mutants of a diploid gene, fourth round mutagenized V79-4 cells were infected with MLV-Barnase to isolate clonal populations that were resistant to retrovirally mediated infection. By initially utilizing a simpler retrovirus, our goal was to eventually isolate and distinguish between clones that are resistant to only Moloney virus not lentivirus, or resistant to both viruses. To isolate clonal populations resistant to retrovirally mediated infection, we repeatedly infected post 4^{th} round mutagenized V79-4 cells for 20 days to ensure that each cell was target by at least one retroviral particle. Each day cells were rinsed with media and virus generated that day was added. After selection using MLV-barnase, 300 colonies were visible on ten 10 cm plates and 96 colonies were isolated and

Table 2-1: Rate of appearance of 6TG and DAP resistant colonies.

	6-Thioguanine Resistant (HPRT-) Colonies per 10^7 cells	Diaminopurine Resistant (APRT-) Colonies per 10^7 cells
Spontaneous	3	0
Post Round 1 Mutagenesis	109	0
Post Round 2 Mutagenesis	not assayed	1
Post Round 3 Mutagenesis	not assayed	17
Post Round 4 Mutagenesis	not assayed	22

expanded for further analysis. Initial analysis involved infecting these 96 clones with a lentiviral vector transducing GFP (CSII-EF-EFGP). Based on visual inspection by multiple individuals, the percentage of cells infected was evaluated and compared. Based on the results of this visual quantification, clones 31 and 67 were observed to have decreased numbers of cells expressing GFP when compared to the other 94 clones and wild-type V79-4 cells. These two clones were further analyzed for their resistance phenotypes. Clone 31 is considered in this chapter and clone 67 is characterized in detail in the following chapter.

Confirming the Retroviral Resistance phenotype

Initially, to confirm that we had isolated a clonal population, clone 31 was plated at low densities to generate sub-clonal populations. Ten subclones of 31 were isolated and tested further and all were stable for the resistance phenotype observed in the parental line (data not shown). Clone 31-2 was used in all subsequent experiments. First, growth curve analysis was conducted on 31-2 (Fig. 2-5; squares) and the parental V79-4 cell lines (Fig. 2-5; diamonds). We observed a slightly retarded growth rate of 31-2 compared to the parental V79-4. At 48 hours, the cell density of 31-2 cultures was 97% of V79-4, 59% at 72 hours, 70% at 84 hours, and 79% at 108 hours. We concluded that the delay in the growth rate cannot completely account for the resistance phenotype observed in 31-2 since the resistance phenotype is observable for both retrovirally mediated transduction and lentivirally mediated transduction (discussed in greater detail below).

Next, we needed to determine if transcription was simply suppressed in the mutant cell line leading to a resistance phenotype. To test for this, both V79-4 and 31-2

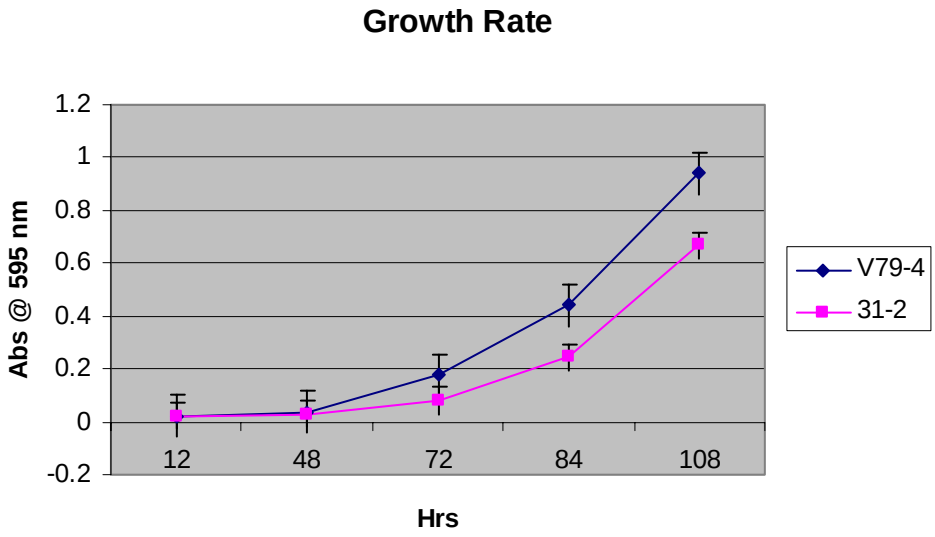

Growth Rate
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
Abs @ 595 nm
12
48
72
84
108
Hrs
V79-4
31-2

Figure 2-5

31-2 cells have a slight growth delay.

General cell density of unperturbed V79-4 (diamonds) and 31-2 (squares) were compared over 106 hours. Abs- absorbance; Hrs- hours; nm- nanometers.

were separately transfected and infected with a lentiviral vector expressing the blasticidinresistance gene and then selected for gene expression with continuous blasticidin treatment (Figure 2-6). Blasticidin resistant colonies were counted after 20 days. If 31-2 had a deficiency in transcription, both the transfection and the infection rates of the resistant cell line would be identical. Although, 31-2 is slightly reduced in its transfection efficiency as compared to V79-4 (Fig. 2-6, 87% for 31-2 normalized to V79-4 values), the reduction in infection rate is much greater (Fig. 2-6, 7% of V79-4 values). The reduction in transfection efficiency is insufficient to account for the reduced level of infectivity. The disparity in relative transfection and infection rates indicates that the transcription machinery of 31-2 was intact and therefore not the cause of the resistance phenotype. The mode of delivery of the transgene is causing the reduction in blasticidin resistance.

To ensure that the resistance phenotype observed in 31-2 was not the result of resistance to VSV-G mediated entry, retroviral vector was generated pseudotyped with an alternative envelope, the MLV amphotrophic 10A1 envelope. The MLV amphotrophic 10A1 facilitates entry via one of two receptors, Pit-1 (Glvr-1) or Pit-2 (Ram-1) (Miller and Chen, 1996; Miller and Miller, 1994), while VSV-G facilitates entry via a fusion event mediated by phospholipid components of the membrane (Mastromarino et al., 1987). Lenti-luciferase pseudotyped with 10A1 was generated and used to infect both 31-2 and the parental V79-4 cell line. 31-2 showed a similar level of resistance (Fig. 2-7, 11% normalized to parental) when using an amphotrophic envelope as compared to using the VSV-G envelope (Fig. 2-6). Thus, the resistance observed in 31-2 is not envelope specific.

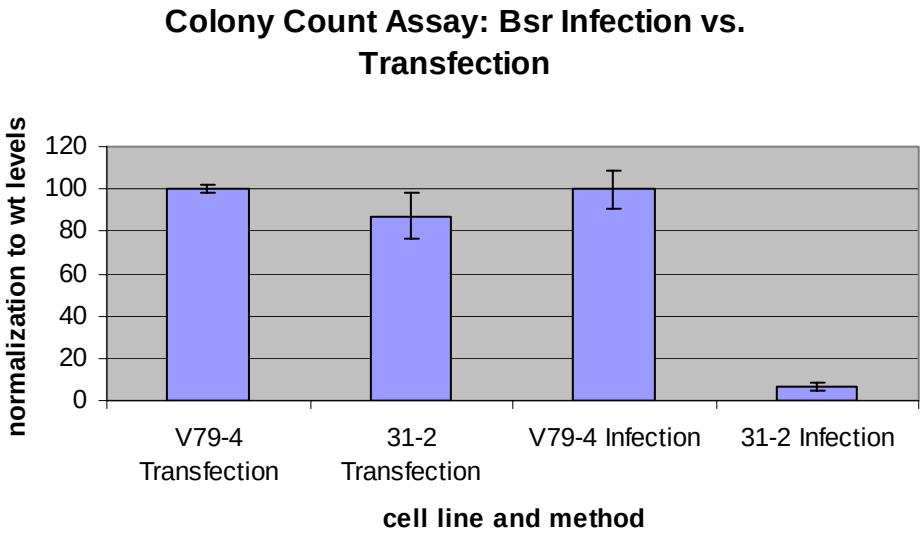

Colony Count Assay: Bsr Infection vs. Transfection
normalization to wt levels
120
100
80
60
40
20
0
V79-4 Transfection
31-2 Transfection
V79-4 Infection
31-2 Infection
cell line and method

Figure 2-6

Mechanism of gene delivery is critical for the resistance phenotype of 31-2 cells. V79-4 and 31-2 cells were either transfected with a viral plasmid conferring resistance to blasticidin or infected with the same virus conferring resistance to blasticidin. The numbers of blasticidin resistant colonies are presented as the number of 31-2 colonies relative to the number of wildtype V79-4 colonies. Each data point represents four different plates that were averaged to generate a final colony count. For the transfection experiments, a large batch of transfection mix was generated and used to transfect both V79-4 and 31-2. For the infection experiments, the same pool of CSII-Bsr virus was used to infect both V79-4 and 31-2. Bsr- blasticidin resistance; wt- wildtype.

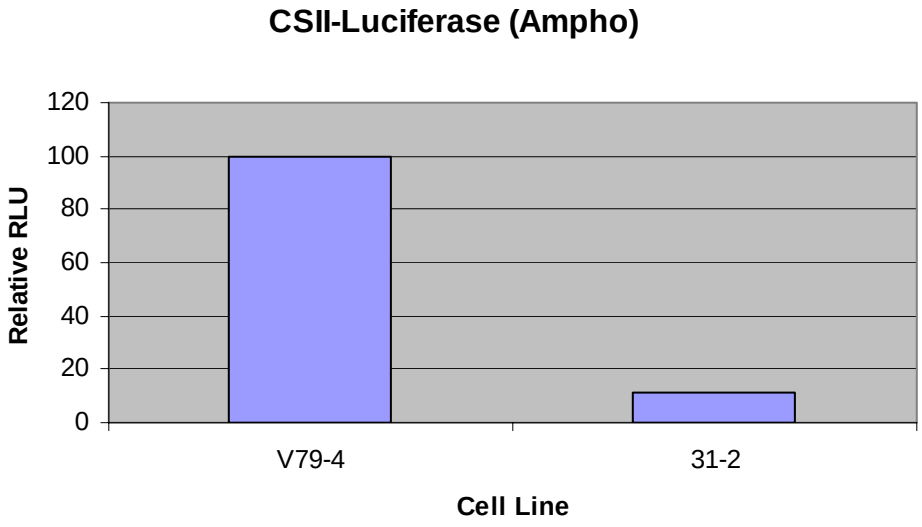

CSII-Luciferase (Ampho)
120
100
80
60
40
20
0
Relative RLU
V79-4
31-2
Cell Line

Figure 2-7

31-2 resistance phenotype is independent of envelope protein species.

Pseudotyping with the Ampho envelope 10A1 does not change the resistance phenotype and the rate of infectivity is comparable with virus pseudotyped with the VSV-G envelope protein (Fig. 2-6). Ampho- Amphotropic 10A1; RLU- relative light units; VSVG- vesicular stomatitis virus g-protein.

Characterization of the Resistant Clone

Once we determined that the resistance phenotype of 31-2 was not the result of a transcriptional defect or an entry defect, we quantified the resistance phenotype using several assays and different multiplicities of infection (m.o.i.). We infected both V79-4 and 31-2 cells with a lentivirus encoding luciferase (CSII-luciferase) varying the m.o.i. over four logs. 31-2 consistently showed a 5 to 10 fold reduction in infectivity as compared to the parental V79-4 cells (Fig. 2-8). Since this reduction was seen across four orders of magnitude, it is likely that the resistance phenotype in 31-2 is not satuarable. This is in contrast to *Fv1* (Decleve et al., 1975; Pincus et al., 1975) and Trim5α (Cowan et al., 2002) mediated resistance, both of which can be overcome at high m.o.i.

Next we transduced both 31-2 and V79-4 with a lentiviral vector encoding GFP (CSII-EF-EGFP) and separately transduced with a Moloney vector encoding GFP (pCLMFG-EGFP). These assays used viral concentrations at an m.o.i. of one and two (viral titer was determined by FACS analysis on HeLa cells). 31-2 is similarly resistant to both Moloney and lentiviral transduction and 31-2 does not appear to be saturable at a higher dose (Fig. 2-9). As assayed by GFP expression using FACS, 31-2 is five fold less infectable compared to its parental wildtype cell line V79-4. Thus, 31-2 is less infectable than V79-4 as measured using different reporters. Hence, resistance to viral transduction is not dependent on the reporter. The results obtained from the transfection/transduction experiment (Fig. 2-6) as well as the alternative envelope assay (Fig. 2-7) suggest that the block to infection in 31-2 is post-entry.

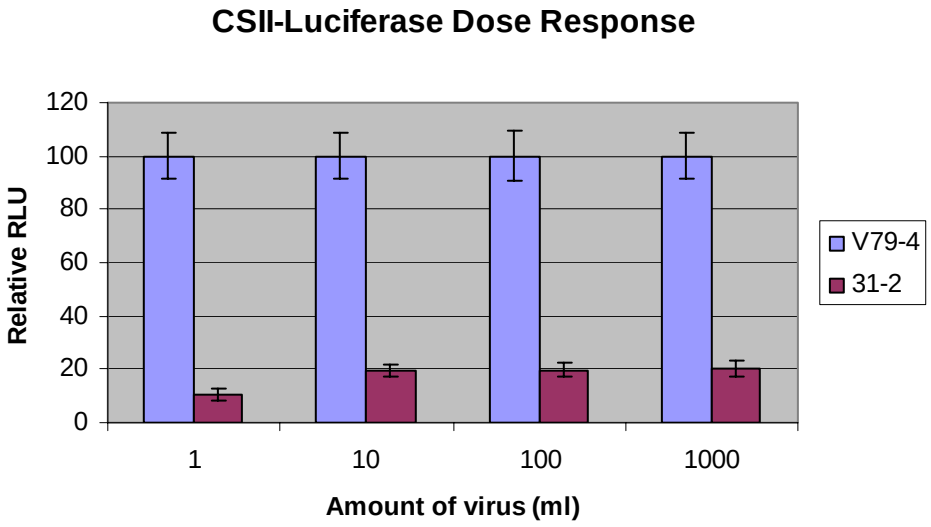

CSII-Luciferase Dose Response
Relative RLU
120
100
80
60
40
20
0
1
10
100
1000
Amount of virus (ml)
V79-4
31-2

Figure 2-8

31-2 infection resistance is not dose dependent.

V79-4 and 31-2 cells were infected the with same batch of CSII-luciferase lentiviral vector over four orders of magnitude, and 31-2 is consistently 5 to 10 fold resistant to retrovirally mediated transduction as compared to wild-type V79-4. RLU- relative light units.

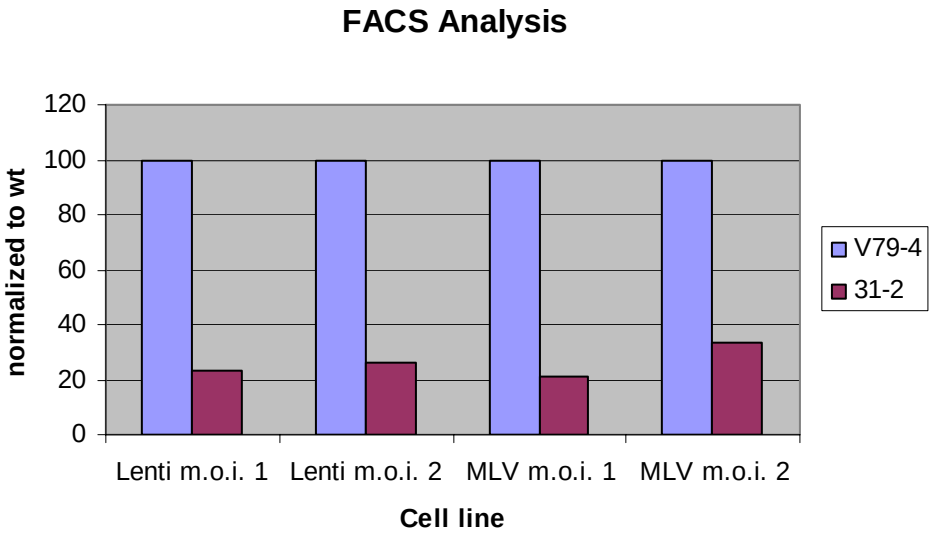

FACS Analysis
120
100
80
60
40
20
0
normalized to wt
V79-4
31-2
Lenti m.o.i. 1
Lenti m.o.i. 2
MLV m.o.i. 1
MLV m.o.i. 2
Cell line

Figure 2-9

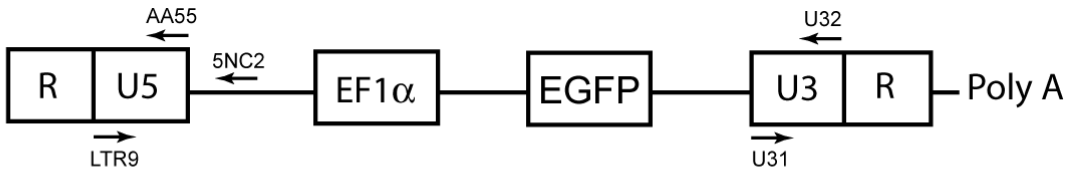
31-2 resistance phenotype is not retroviral specific.

31-2 is resistant to both a lentiviral vector and a Moloney (MLV) based vector encoding EGFP. Flow cytometry analysis was performed 72 hours post infection and relative rates of infection are indicated. 31-2 cells are less infectable than the parental V79-4 cell line and the level of EGFP expression is also reduced (as quantified by Geometric Mean of fluorescence intensity; data not shown). FACS- flow assisted cell sorting; MLV- murine leukemia virus; wt- wildtype.

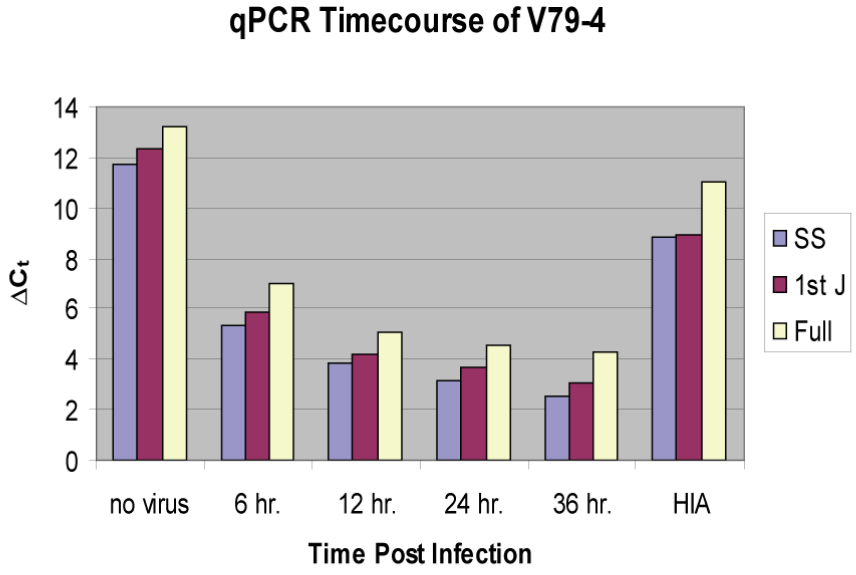
In order to pinpoint the stage at which the block to infection occurs in 31-2 cells, qPCR was conducted on post-infection DNA extracts from both V79-4 and 31-2 cells to quantify the levels of viral genome resulting from the process of reverse transcription (RT). V79-4 and 31-2 were infected with CSII-EF-EGFP vector at an m.o.i. of 0.5 (as titered on HeLa cells) and cells were harvested at 6, 12, 24, and 36 hours post infection for qPCR quantification of reverse transcribed viral genomes. PCR primers were used to quantify the amount of negative strand strong stop product (SS), product generated after the first jump (1^{st} J), and late or end stage products (full) and cell extracts were normalized to a β-actin control (Zack et al., 1990). Fig. 2-10A illustrates the locations of the PCR primers used on the viral transcript for qPCR analysis: LTR9 and AA55 detect SS; U31 and U32 detect 1^{st} J; 5NC and LTR9 detect full.

The qPCR results for V79-4 (Fig 2-10B) and 31-2 (Fig. 2-10C) indicate that the block to infection is after the completion of reverse transcription. Note that a lower ΔC_t value corresponds to a greater amount of RT derived product present in the sample. There does not appear to be a block at the level of reverse transcription, since all the RT derived products appear at all time points and as expected the amount of RT derived products increases over time (Fig. 2-10). Two controls (the no virus control and the heat inactivated virus control) did not show an increase in any product. The amount of RT derived product in 31-2 does not vary when compared to the amount of RT derived product in V79-4. Additionally, the rate of appearance of RT derived product does not vary between cell lines. Since the qPCR data does not show any difference between 31-2 and V79-4 we conclude that the block to infection must occur after the completion of reverse transcription.

A.


AA55
5NC2
U32
R
U5
EF1α
EGFP
U3
R
Poly A
LTR9
U31

B.


qPCR Timecourse of V79-4
ΔCt
14
12
10
8
6
4
2
0
no virus
6 hr.
12 hr.
24 hr.
36 hr.
HIA
Time Post Infection
SS
1st J
Full

C.

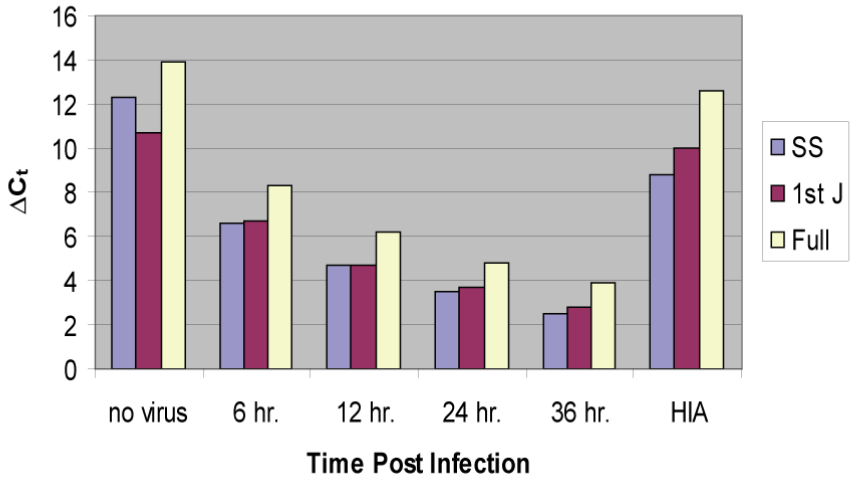

qPCR Timecourse of 31-2
ΔCt
16
14
12
10
8
6
4
2
0
no virus
6 hr.
12 hr.
24 hr.
36 hr.
HIA
Time Post Infection
SS
1st J
Full

Figure 2-10

V79-4 and 31-2 exhibit no difference in the progression of reverse transcription during retroviral infection.

(A) Location of PCR primers used for qPCR analysis along the lentiviral RNA. The virus used to infect V79-4 and 31-2 for qPCR analysis was CSII-EF-MCS-EGFP, a lentiviral vector that encodes for EGFP driven by the EF1α promoter. Primers AA55 and LTR9 were used to quantify strong stop product (SS). Primers U31 and U32 were used to quantify 1st Jump product (1st J). Primers 5NC2 and LTR9 were used to quantify late or end stage product (full). The position of each primer used is indicated. (B) Quantifying strong stop (SS), first jump (1st J), and late or end stage (full) products over time in V79-4 cells. (C) Quantification of RT products over time in 31-2 cells. For both (B) and (C) the amount of product is expressed relative to the amount of β-actin transcript detected (ΔC_t). Cells where no virus was added or heat inactivated virus was added (HIA) served as negative controls. Control samples were harvested at 36 hours. Note that a decrease in the ΔC_T corresponds to an increase in viral DNA. 1st J- 1st jump product; C_t- cycle threshold; EF1α- elongation factor 1α; EGFP- enhanced green fluorescent protein; full- late or end stage product; HIA- heat inactivated; hr- hour; SS- strong stop product.

Discussion

This study presents a novel toxic retroviral vector that does not require the use of a prodrug. The toxic gene barnase and its potent inhibitor barstar are both derived from *Bacillus amyloliquefaciens*. This toxic vector is capable of inducing cell death in a variety cell types derived from different species. We generated this retroviral vector at high titers without excessive toxicity to the packaging cell line because an inhibitor, barstar, also derived from *Bacillus amyloliquefaciens*, was also expressed in the packaging cell. Retroviral vectors transducing the toxic barnase gene were used to infect mutagenized V79-4 cells and identify cells that were resistant to retroviral infection. One resistant cell line, 31-2, was isolated and further characterized. This cell line is approximately 5-10 fold less infectable than its parental cell line. The resistance phenotype is cannot be accounted for by envelope variability, dose dependency, or virus specificity. qPCR analysis shows that the block to infection in 31-2 occurs after the completion of reverse transcription but before expression of the gene encoded by the retroviral vector. This study shows that barnase based retroviral vectors can efficiently kill a wide variety of cell types and can be effectively used to conduct loss of function genetics in cell culture to elucidate the mechanism of retroviral infections.

Barnase: an ideal selection agent

We have identified barnase as a potent new suicide gene. The major advantage of barnase is that the toxic product is the gene itself and therefore no prodrug is required. Another toxic gene product, the A subunit of diphtheria toxin (DT-A) does not require any cofactor or pro-drug (Qiao and Caruso, 2002), similar to barnase. However, it has also been shown that resistance to DT-A can be conferred by a single mutation in codon

715 of human Elongation Factor 2 (EF-2, (Ivankovic et al., 2006)). EF-2 at codon 715 contains a histidine residue which is post-translationally modified to diphthamide, and if this histidine is converted to leucine, methionine, asparagine or glutamine, resistance to DT-A is conferred (Ivankovic et al., 2006). However, this potential limitation does not prevent DT-A from being used in a wide variety of studies to kill cancer cells both *in vivo* (Dang et al., 2007; Evens et al., 2007) and in cell culture systems (Frankel et al., 2000; Qiao and Caruso, 2002). With further study, barnase based killing systems may be able to be used in a similar manner with little toxicity and high efficacy.

The only additional component necessary to create high titer barnase vector is its inhibitor, barstar. This inhibitor is required in the packaging cell line and is not necessary to facilitate the toxic properties of barnase during infection. Although barnase encoding virus could be generated without the presence of barstar in the packaging cells (Fig 2-3; diamonds), greater toxicity is observed on infection across all dilutions and cell types when virus was generated in the presence of barstar (Fig. 2-3, squares), indicating production of higher titer vector preparations. It was quite puzzling that any toxic virus could be generated in the absence of the inhibitor barstar since barnase protein is being produced in the packaging cell lines. However, it is possible that the rapid assembly of virus particles, even in the absence of barstar, allowed for the production of a certain amount of virus before barnase based toxicity prevented all virus production or killed the packaging cell line. This is consistent with what we observed since it took approximately three days to observe complete killing with both MLV and lentiviral based barnase vectors. Finally, the barstar/barnase system can be used to create stable, long-term packaging cell lines that could continuously produce high titer retroviral vectors. By

generating a stable cell line already expressing barstar, barnase virus can be continuously produced without any toxic effects to the packaging cell line. We conclude that the presence of barstar, the inhibitor of barnase, allows for the generation of high titer retroviral vectors and ultimately can be used to create stable packaging cell lines for high volume production of vectors.

One potential shortcoming with some current suicide gene therapies is the need for a non-toxic prodrug which is converted to a toxic product by the suicide gene. For example, the most commonly used pro-drug approach uses the HSV-*TK* gene and the prodrug, either gancylovir or acyclovir, that is converted to a toxic product by HSV-*TK* (Mar et al., 1985; Reid et al., 1988). To facilitate cell death, either the prodrug and gene product must be present in the same cell or the cells must form gap junctions in order to allow prodrug, gene product, or toxic product to flow from one cell to another. Although utilization of this bystander effect is advantageous under some circumstances, it may not be ideal for all circumstances. Additionally, sometimes internalization of both the pro-drug and the gene is not 100% efficient or there is resistant to the pro-drug and gene product combination (Sturtz et al., 1997). By utilizing barnase one can eliminate some problems associated with both pro-drug and gene product internalization or resistance, by simply using a different killing mechanism that does not require a pro-drug. Both the MLV and lentiviral based barnase vectors can kill cells efficiently, but the saturation point of MLV based toxicity is consistently higher than that of its lentiviral based counterpart, indicating a greater overall survival rate (Fig. 2-3). The differences observed between MLV and lentiviral based vectors are expected because lentiviruses can infect non-dividing lymphocytes (Zack et al., 1990) and other non-dividing cell types

(Yamashita and Emerman, 2006), while MLV is limited to infecting cells which are undergoing mitosis (Roe et al., 1993). Although results using both MLV- and lentiviral-based barnase vectors indicated saturation points, when the same set of cells were repeatedly infected there was no cell survival, indicating that with a high enough m.o.i., cell survival can be reduced to zero. The different levels of cell death between cells types could be caused by a variety of factors: variable expression from MLV LTRs and the EF1α promoter that drives the expression of barnase or differential levels of initial infectivity. Regardless of the variability observed between the MLV- and lentiviral-based vectors as well as the variability across cell types, we demonstrate that barnase based retroviral vectors can kill a wide variety of cell types at high efficiency.

Applications of barnase selection

Barnase can be used to target cells for destruction in a number of ways, each potentially creating greater and greater specificity of targeting. When combined with tissue specific envelopes and/or tissue specific promoters, the barnase retroviral vector can be used to effectively infect and kill a specific cell type. Non-targeted cells would avoid destruction. For example, advances in targeted delivery of retroviruses have lead to the discovery of tissue specific envelopes that specifically target carcinoembryonic antigen (CEA) expressing cells (Kuroki et al., 2000) and metastatic melanoma cells (Morizono and Chen, 2005). Various cancer specific promoters have also been identified including probasin, human telomerase reverse transcriptase, survivin, ceruloplasmin, HER-2, and osteocalcin (Lo et al., 2005). By simply replacing the pantrophic envelope VSV-G with a targeting envelope and the constitutive active promoter found in lenti-barnase (EF1α) with a cancer specific promoter, one can create a retroviral vector that

can target a specific cancer at both the entry level and at the protein expression level. This leads to greater specificity of killing and reduced off-target killing. Given these two advantages, targeted barnase based retroviral vectors are an ideal suicide gene system when the bystander effect is either not needed or unavailable.

In this study, novel barnase based retroviral vectors were used to identify mutagenized cells that were resistant to retroviral based transduction. Given that this resistance phenotype was not detected when the mutagenized 31-2 cells were transfected with the retroviral vector plasmid, the 31-2 cells must misexpress or harbor a mutation in a host cell protein that is necessary for the retrovirus to complete its life cycle. 31-2 has a resistance phenotype that must be post entry since substituting an amphotrophic envelope for VSV-G does not alter the resistance phenotype (Fig. 2-7). 31-2 also produces equivalent levels of reverse transcribed product detected by qPCR compared to its wildtype parental V79-4 cells (Fig. 2-10). Taken together, 31-2 resistance lies at a point post reverse transcription. 31-2 resistance could be at a step associated with the formation of a preintegration complex or at integration itself. It is likely the resistance phenotype is not at the level of nuclear import as both MLV-, which does not rely on nuclear import for integration (Roe et al., 1993), and lenti-based retroviral vectors are resistant to infection in 31-2. Further studies will be necessary to identify where the block to infection actually occurs and the host protein(s) responsible for this phenotype. The use of barnase based retroviral vectors to identify mutant cell lines for host cell proteins in cell culture is a powerful technique to elucidate the pathway for HIV-1 infection. By understanding the HIV infective pathway and the host cell factors

necessary to complete that pathway, it may be possible to create new potential drug targets and develop new methods to prevent infection or progression of HIV and AIDS.

CHAPTER 3

Isolation, characterization, and complementation of a cell line resistant to retrovirally mediated transduction

Introduction

HIV-1 infection of human cells requires significant interaction between cellular and viral factors. All the viral factors encoded by HIV are contained within an mRNA transcript that encodes fifteen distinct proteins (Frankel and Young, 1998). These fifteen proteins are insufficient to complete the life cycle of HIV. Consequently, HIV behaves as an obligate intracellular parasite by harnessing cellular machinery to complete its life cycle. A number of cellular proteins have been identified that interact with HIV at various stages of the HIV life cycle. HIV Gp120 interacts with a cell surface receptor CD4 and co-receptors CCR5 or CXCR4 to initiate viral particle binding to the host cell (Deng et al., 1996; Deng et al., 1997; Dragic et al., 1996), Kif4 and the microtubule network may facilitate HIV and HIV component trafficking (Kizhatil and Albritton, 1997; McDonald et al., 2002; Tang et al., 1999), and LEDGF (lens epithelium-derived growth factor) stabilizes the HIV PIC (pre-integration complex) and facilitates HIV integration (Cherepanov et al., 2003; Maertens et al., 2004; Maertens et al., 2003). Furthermore, several cellular pathways have been identified that may be involved in HIV infection including cell cycle progression (Cannavo et al., 2001) and the endoplasmic reticulum degradation pathway (Courageot et al., 1999). Identification of interacting host cell proteins may generate attractive targets for new HIV therapies as they do not undergo the high mutation rate of HIV proteins.

Although it has been very informative to identify the host cell factors and pathways involved in infection, the recent identification of two host cell antiviral proteins may lead to more effective antiviral therapies. APOBEC3G is well characterized as a cytidine deaminase that incorporates into virions during viral production and induces deamination of deoxycytidine to deoxyuridine (Harris et al., 2003). This deamination results in hypermutations from G to A during the process of reverse transcription of the viral mRNA, leading to some degradation of the viral genome and partial inhibition of HIV-1 infection (Harris et al., 2003; Mangeat et al., 2003). However, this host defense is overcome by the HIV accessory protein Vif which targets APOBEC3G for degradation via the proteasome (Yu et al., 2003). APOBEC3G was an important discovery because it demonstrated that humans have a host cell protein that not only interacts with HIV but also has antiviral properties. By understanding the mechanism of APOBEC function and activity, we may be able to develop antiretroviral therapies that mimic its actions, enhance its activity, or prevent its destruction.

A second antiviral host protein, Trim5α, was also discovered in the last decade. Rhesus monkey Trim5α (previously known as *Lv1* (Cowan et al., 2002)) was identified as the gene responsible for inhibiting HIV infections in non-human primates (Stremlau et al., 2004). Trim5α was shown to inhibit HIV-1, but not SIV_{MAC} in Old World Monkeys (old world monkeys are consequently resistant to HIV infections). Further work led to the conclusion that *Ref1*, a gene that conferred resistance to N-trophic murine leukemia virus (MLV) in several human cell lines (Besnier et al., 2003; Towers et al., 2000), was human Trim 5α (Yap et al., 2004). *Lv1* and *Ref1* were shown to be species specific versions of Trim5α, each capable of efficient inhibition of viruses that do not normally

target their own species (Hatziioannou et al., 2004). Further investigation into the Trim evolutionary tree found African Green Monkey Trim5α, which is capable of inhibiting divergent retroviruses from human, murine, primate, and equine origins (Keckesova et al., 2004). Although the complete mechanism of Trim5α function has not been fully elucidated, Trim5α proteins present in the cytoplasm recognize sites within the viral capsid proteins and interfere with the uncoating process, thereby preventing completion of the HIV lifecycle (Sebastian and Luban, 2005; Stremlau et al., 2006). It has been proposed that Trim5α may be involved in a proteasome-degradative pathway (Wu et al., 2006) and recent evidence showing Trim 5α to be a RING-finger-type E3 ubiquitin ligase targeted by self-ubiquitination as well as ubiquitination by Ro52 furthers this hypothesis (Yamauchi et al., 2008). Discovery of the Trim5 family of restriction factors expanded our understanding of the genes humans cells have developed to inhibit retroviral restrictions, opening the door to the possibility that other such genes exist and giving us more candidates for antiretroviral therapies.

Trim5α was identified in a gain of function genetic screen utilizing a cDNA library generated from primary rhesus monkey lung fibroblasts (Stremlau et al., 2004). However, the majority of cellular HIV-1 interacting proteins have been identified via biochemical approaches, either yeast two hybrid or direct protein-protein interactions (Kim et al., 1998; Luban et al., 1993; VerPlank et al., 2001). While loss of function somatic cell genetic screens have not been commonly used to analyze the viral infection pathway, loss of function genetic screens have been used to identify genes involved in DNA repair (Thompson, 1989) and interferon signaling (Stark, 1997). One study utilized loss of function genetics to analyze viral infections. In this study, heavily mutagenized

Rat-2 cells deficient for thymidine kinase were challenged with a MLV based vector encoding the herpes simplex 1 virus thymidine kinase (HSV-*TK*) gene (Gao and Goff, 1999). Cells which were successfully transduced were killed in the presence of the prodrug trifluorothymidine (TFT). TFT is a toxic thymidine analogue that is activated by HSV-*TK* dependent phosphorylation. Upon activation, TFT can be utilized by the cell during DNA replication where it causes termination of replication and cell death. The authors successfully isolated two clones that were refractory to infection by ten to 100-fold as compared to the parental cell line. Further analysis of one of the clones led to the identification of FEZ1, which when overexpressed caused retrovirus restriction (Naghavi et al., 2005). Unfortunately, the mechanism by which the overexpression rescued the mutant phenotype was not elaborated. This study illustrated that it is possible to isolate clonal populations resistant to HIV-1 infection using a loss of function genetic screen.

In this study, we present a loss of function genetic screen that has identified a novel protein we have named Modulator of Retroviral Infection, MRI. The previous chapter validated this approach as a method to generate mutant cell lines resistant to retroviral infection (Agarwal et al., 2006). To identify MRI, a cell line was mutagenized with ICR-191, a chemical mutagen, and then challenged by a retroviral vector encoding a toxic gene product, barnase. Clones resistant to infection survived barnase selection while those still susceptible were killed. One clone, 67-1, was isolated and characterized further. This clone had a block to infection post entry but before the initiation of reverse transcription and possibly at the stage of uncoating. The block can be reversed with the use of a proteasome inhibitor MG-132 which stabilizes p24 levels in the mutant 67-1 line. We further analyzed the clone by Genome Functionalization through Arrayed cDNA

Transduction (GFAcT) (Chanda et al., 2003; Conkright et al., 2003) to identify a gene conferring the resistance by either complementation or by overexpression suppression. Expression of MRI, the gene identified by GFAcT, restores infectivity in 67-1, and suppression of MRI recapitulates a resistance phenotype. Although little is currently known about MRI, further analysis may lead to a greater understanding of its role in retroviral infections and another potential target for HIV drug therapy.

Results

We have previously shown (Chapter 2 and (Agarwal et al., 2006)) that a mutagenesis strategy in V79-4 cells is effective in generating null mutants for both single and double copy genes and that toxic retroviral vectors based on Barnase can effectively kill cells permissive to retroviral infection, while allowing those resistant to infection to survive. Combining the mutagenized cells with the toxic retroviral vector we were able to identify cell lines resistant to retroviral infection. We have previously characterized a cell line resistant to retrovirally mediated transduction, 31-2, and this study focuses on a second resistant cell derived from that screen, 67-1.

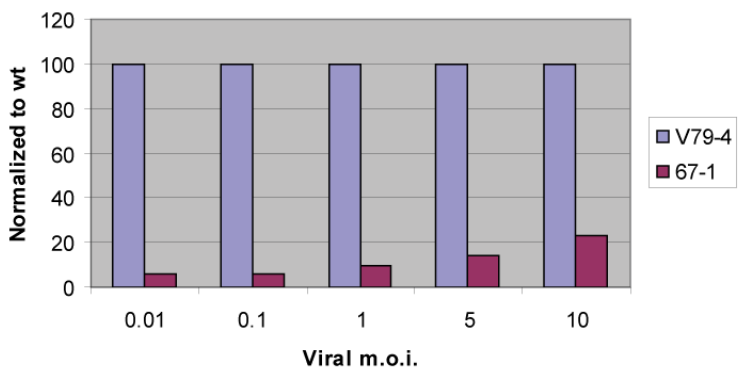
Determining the Level of Resistance

In order to determine the level of infectivity in our mutant 67-1 cells relative to the parental V79-4 cells we standardized our infection conditions using multiplicity of infection (m.o.i.) measurements. Multiplicity of infection is the ratio of infectious agents to infective targets. For example, an m.o.i. of one would correlate to one virion per cell. In order to calculate the m.o.i., large batches of virus were generated, frozen into aliquots, and several different volumes of a newly thawed aliquot of virus were used to challenge 1 x 10^5 HeLa cells. Subsequently, flow cytometry was used to determine the proportion of

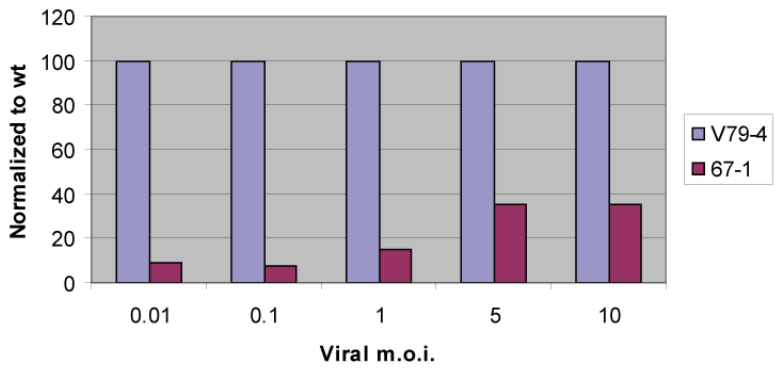
EGFP positive cells and we calculated the number of infective virus particles in the original aliquot.

To determine the level of resistance in the clone, both the V79-4 and 67-1 lines were challenged with a lentiviral vector encoding EGFP (CS-CDF-CG-PRE), a lentiviral vector encoding luciferase (CSII-luciferase), or an Moloney Murine Leukemia (MLV) based vector encoding EGFP (CLMFGv2ΔNcoI-GFP). 67-1 is resistant to both lenti and MLV based retroviral vectors, and this resistance is apparent at both a low and high m.o.i. (multiplicity of infection) (Fig. 3-1A and 3-1B). At an m.o.i. of 0.01 and 0.1 the lentiviral resistance was 20 fold and at an m.o.i. of 1 the resistance was 12 fold compared to the parental V79-4 line. When both V79-4 and 67-1 cells were challenged with virus ranging from an m.o.i. of .01 to an m.o.i. of 10, 67-1 continued to be resistant to retrovirally mediated transduction while V79-4 remained susceptible through all four orders of magnitude. This is in contrast to the innate host cell defense provided by the Trim 5α restriction factors, *Ref1* and *Lv1*, that are saturatable and can be overcome by a high m.o.i. (Hatziioannou et al., 2003). It is worth noting that at a high m.o.i. of 10, 67-1 is only five fold resistant to lentiviral vectors and only three fold resistant to MLV retroviral vectors as normalized to V79-4 rates of infections. However, the data does account for multiple infection events. At higher m.o.i., EGFP expression in the V79-4 cells was brighter as measured by flow cytometry and quantified by geometric mean of fluorescence intensity (data not shown). Higher levels of fluorescence within individual cells indicated that the wildtype cells were multiply infected and therefore expressing much higher levels of EGFP. The mutant 67-1 cells were consistently less bright across

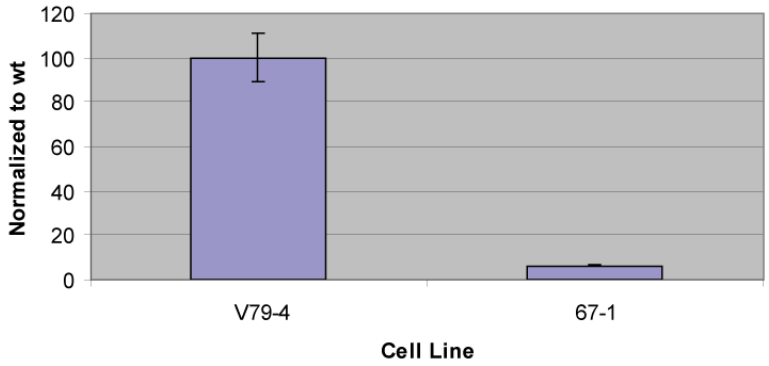
A


Lentiviral Vector Infections for Saturability
Normalized to wt
120
100
80
60
40
20
0
0.01
0.1
1
5
10
Viral m.o.i.
V79-4
67-1

B


MLV Vector Infections for Saturability
Normalized to wt
120
100
80
60
40
20
0
0.01
0.1
1
5
10
Viral m.o.i.
V79-4
67-1

C


CSII-Luciferase Infections
Normalized to wt
120
100
80
60
40
20
0
V79-4
67-1
Cell Line

D

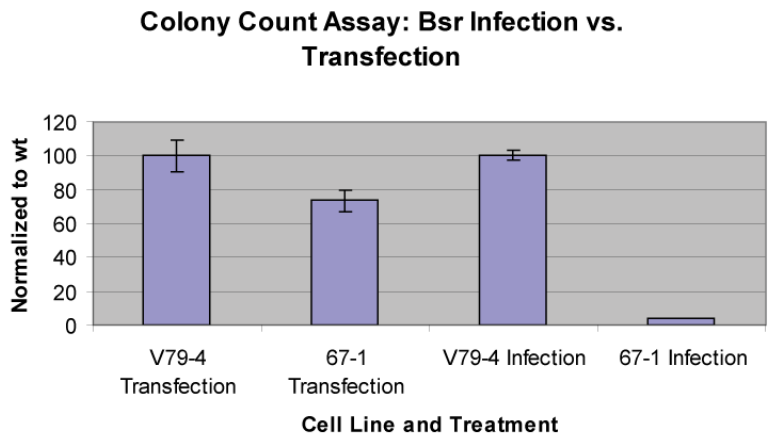

Colony Count Assay: Bsr Infection vs. Transfection
Normalized to wt
120
100
80
60
40
20
0
V79-4 Transfection
67-1 Transfection
V79-4 Infection
67-1 Infection
Cell Line and Treatment

Figure 3-1

67-1 is resistant to retrovirally mediated transduction and is not readily saturable. (A) 67-1 is resistant to lentivirally mediated transduction over four orders of magnitude. (B) 67-1 is resistant to MLV retrovirally mediated transduction over four orders of magnitude. (C) 67-1 is resistant to retrovirally mediated transduction with luciferase, an alternative reporting system. (D) The resistance in 67-1 is retroviral specific since transfecting a plasmid encoding blasticidin resistance results in a level transfection efficiency comparable to wildtype V79-4. The error bars within the data represent standard deviation. Bsr- blasticidin resistance; MLV-moloney leukemia virus; m.o.i.- multiplicity of infection; wt- wildtype.

all m.o.i. and therefore expressing lower levels of EGFP, suggesting that they were less infectable. Taking into account multiple infections, the extent of the resistance phenotype observed in 67-1 may be underestimated at the high m.o.i when looking only at the percent of cells that are EGFP positive. When luciferase was substituted for EGFP and quantified to look at the amount of overall infection, the fold resistance between 67-1 and V79-4 increased to 36 (Fig. 3-1C). Depending on the assay system utilized and the m.o.i., 67-1 cells are at least 10 fold less infectable than wildtype V79-4 cells (see also below for blasticidin resistance gene transfer).

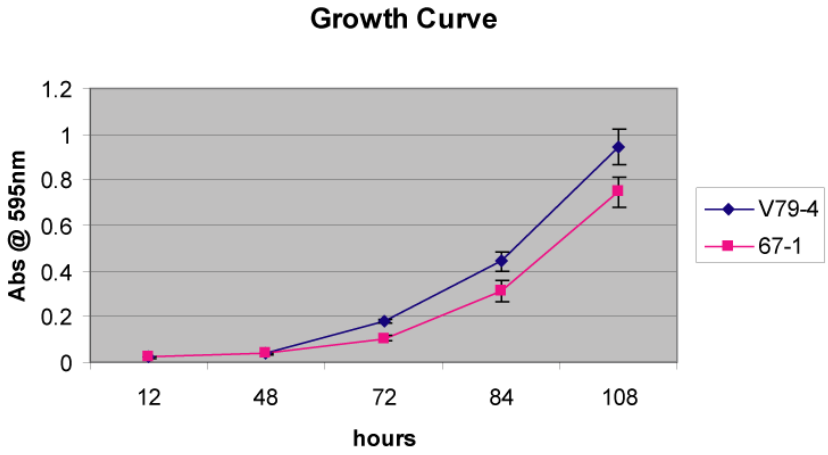
To ensure that the resistance phenotype was a result of a non-productive infection rather then an inhibition of transgene expression, V79-4 and 67-1 were either infected with a lentiviral vector transducing a blasticidin-GFP fusion protein (CSII-Bsr-GFP) or transfected with the viral vector plasmid encoding the same protein. Notably, gene expression for the blasticidin resistance gene is dictated by the same promoter in both delivery mechanisms. When transfection and infection levels are normalized to V79-4 levels, 67-1 yields many more drug resistant colonies upon transfection (70% of wt levels) when compared to an infection (4% of wt levels, Fig. 3-1D). These data indicate that the mechanism of delivery of the transgene was critical since the 67-1 cell line was resistant to the viral infection but not resistant to transfected plasmid. The slight decrease in colonies seen in the transfection can be attributed to variations in transfection efficiencies between cell lines since 67-1 is a mutated clonal derivative of V79-4. We conclude that the resistance phenotype in 67-1 results from reduction in virally transduced gene expression and not simply due to a mutation that impacts expression of our reporter genes.

Characterizing the resistant clone

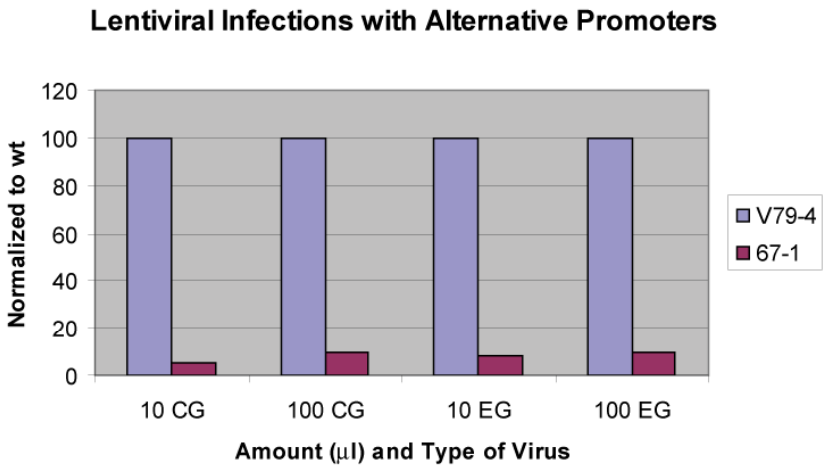
Once the level of resistance of 67-1 was determined using a variety of reporting assays we further characterized the resistance phenotype. We first tested whether or not a difference in growth rate could account for the resistance phenotype observed in 67-1. To accomplish this, growth curves were generated for both cell lines (Fig. 3-2A). Although 67-1 cells have a slightly retarded growth rate compared to the parental V79-4 cells (at 84 hours 67-1 cultures have 70% of the cell density as V79-4 cultures and at 108 hours 67-1 cultures have 79% of the cell density as V79-4 cultures), the reduction in growth rate is insufficient to account for the resistance phenotype. To characterize the resistance further, V79-4 and 67-1 cells were challenged with lentiviral vectors encoding EGFP driven by CMV (CS-CDF-CG-PRE) or EF1α (CS-CDF-EG-PRE), and we discovered that 67-1 is similarly resistant regardless of the promoter used (Fig. 3-2B); supporting our previous conclusion that the resistance phenotype was not at the level of gene expression. These two results indicate that the resistance phenotype of 67-1 is not caused by a reduced growth rate or by promoter variability.

We next determined whether the mutation in 67-1 cells was dominant or recessive because if the mutation was dominant we would need to construct an expression clone cDNA library from 67-1 cells to determine the cause of the resistance phenotype, similar to the method used to discover Trim5α (Stremlau et al., 2004). To determine the type of mutation we conducted cell fusion experiments with membrane labeled V79-4 and 67-1 cells. V79-4 or 67-1 cells were labeled with Oregon Green (stains membranes green) or Vybrant DID (stains membranes blue) and fused by the addition of polyethylene glycol to

A


Growth Curve
Abs @ 595nm
1.2
1
0.8
0.6
0.4
0.2
0
12
48
72
84
108
hours
V79-4
67-1

B


Lentiviral Infections with Alternative Promoters
Normalized to wt
120
100
80
60
40
20
0
10 CG
100 CG
10 EG
100 EG
Amount (µl) and Type of Virus
V79-4
67-1

C

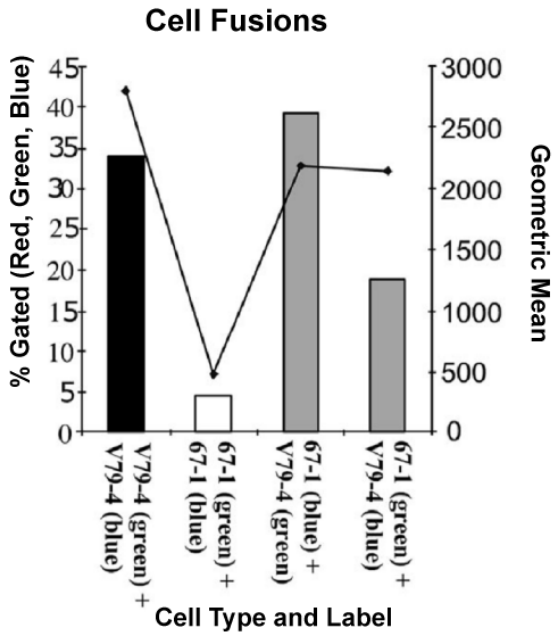

Cell Fusions
% Gated (Red, Green, Blue)
45
40
35
30
25
20
15
10
5
0
3000
2500
2000
1500
1000
500
0
Geometric Mean
V79-4 (blue)
V79-4 (green) + V79-4 (blue)
67-1 (blue)
67-1 (green) + 67-1 (blue)
V79-4 (green)
67-1 (blue) + V79-4 (green)
V79-4 (blue)
67-1 (green) + V79-4 (blue)
Cell Type and Label

Figure 3-2

Further characterization of the resistant clone.

(A) 67-1 has a slightly retarded growth rate relative to V79-4, however it is insufficient to account for the resistance phenotype. (B) The resistance phenotype is not promoter specific as lentiviral vectors that dictate EGFP expression with either CMV (CG) or EF1α (EG) are equally resistant in 67-1. (C) The resistance phenotype in 67-1 is recessive. V79-4 and 67-1 cells were labeled with fluorescent membrane dyes (blue or green) and either self fused or fused to each other using polyethylene glycol. Fused cells were infected with a lentiviral vector transducing dsRed and the extent of infection was determined by flow cytometry. The extent of infection (columns and left y-axis) was determined by determining the proportion of green, blue, and red cells as compared to the cells that are only green and blue. The amount of dsRed expression, which correlates gene expression and multiply infected cells, is quantified as the geometric mean of the red fluorescence (line and right y-axis). The data presented in Figure 3-2C were generated by Patrycja Lech, another member of the lab. Abs- absorbance; CG- lentiviral vector that expresses EGFP from the CMV promoter; EG- lentiviral vector that expresses EGFP from the EF1α promoter, nm- nanometer; wt- wildtype.

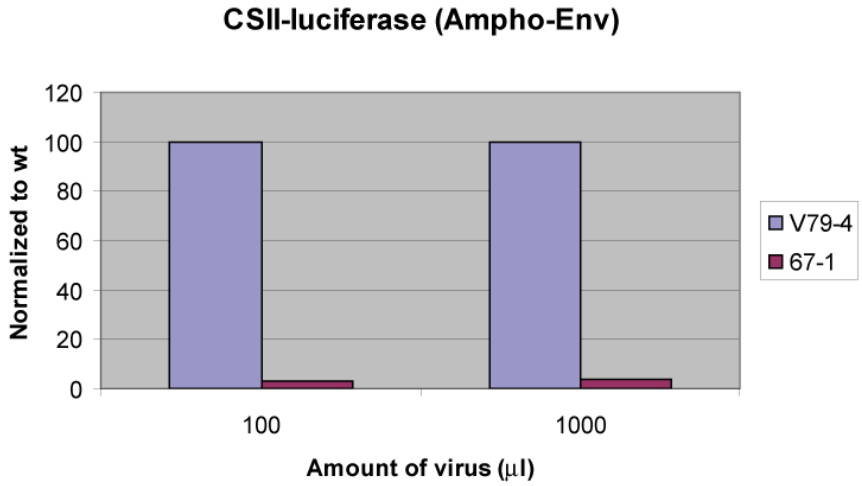
make self-self or 67-1/V79-4 combination homo or hetrokaryons.[1] These fused cells were then infected with a lentiviral vector encoding dsRed. These green, blue, and potentially red labeled cells were then analyzed by fluorescence cytometry for color and intensity (Fig. 3-2C). When resistance levels were calculated for the self-self fusions, 67-1 continued to be resistant to the CSII-EF-dsRed lentiviral vector (4.3% infectable when compared to 34% infectable for the V79-4 self/self fusions). However, when 67-1 cells were fused to V79-4, the resistance phenotype was either completely eliminated (cells were 39.2% infectable when the 67-1 cells were labeled blue and the V79-4 cells were labeled green) or partially eliminated (cells were 18.7% infectable when the 67-1 cells were labeled green and the V79-4 cells were labeled blue). Additionally, the 67-1/V79-4 fused cells expressed dsRed at an intensity comparable to that of wildtype V79-4 (as determined by geometric mean of fluorescence intensity). It is unknown why we consistently observed greater recovery when 67-1 cells were labeled blue and V79-4 cells were labeled green. Given these results, we concluded that the mutation in 67-1 was recessive as fusion with the wildtype V79-4 cells resulted in a fused cell that had greater susceptibility to retrovirus infection as compared to fusion with 67-1.

Determining the stage of the block to infection

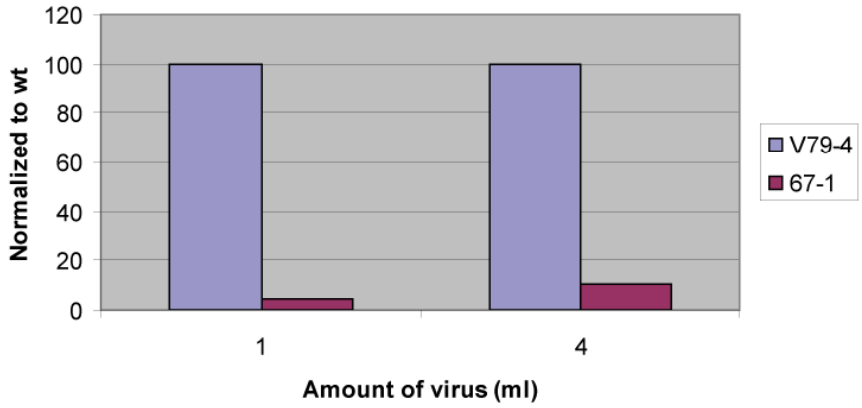
To determine at what stage the block to infection was occurring, we initially examined a lentiviral vector encoding luciferase pseudotyped with the 10A1 amphotrophic envelope (Fig. 3-3A). Utilizing the amphotrophic envelope, we observed a 30 fold decrease in the level of infectivity when normalized to wildtype levels. This is consistent with the amount of resistance observed with a lentiviral vector encoding

[1] Another member of the lab, Patrycja Lech, conducted this experiment.

A


CSII-luciferase (Ampho-Env)
120
100
80
60
40
20
0
Normalized to wt
100
1000
Amount of virus (µl)
V79-4
67-1

B


CSII-GFP (Int- virus) Infection
120
100
80
60
40
20
0
Normalized to wt
1
4
Amount of virus (ml)
V79-4
67-1

C

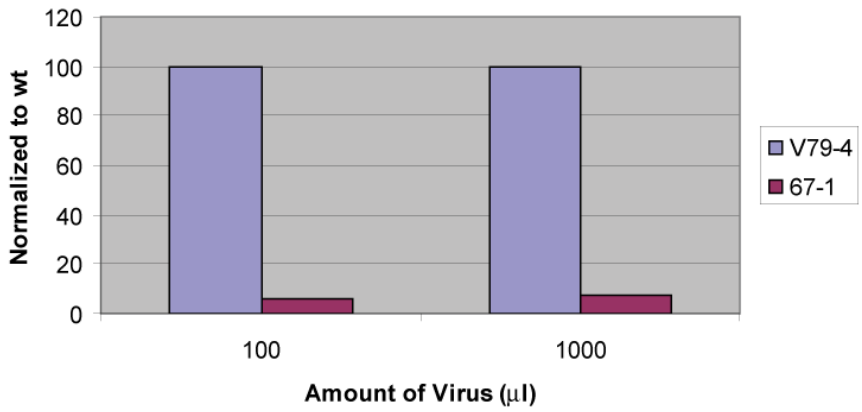

FIV-GFP Infection
120
100
80
60
40
20
0
Normalized to wt
100
1000
Amount of Virus (µl)
V79-4
67-1

Figure 3-3

Isolating the block to infection.

(A) 67-1 remained refractory to lentivirally mediated transduction when the virus was pseudotyped with the 10A1 Amphotropic envelope. A similar infection rate was seen with the VSV-G envelope (Fig. 3-1C), indicating the block to infection was independent of the receptor and mode of entry. (B) 67-1 was resistant to an Integrase (Int-) lentiviral vector, indicating that the block to infection occurred prior to the lentiviral vector integrating into the host cell DNA. (C) 67-1 was resistant to a lentiviral vector closely related to HIV further implying that the resistance is in a pathway common to gammaretroviruses and lentiviruses (MLV, HIV, and FIV). Ampho- amphotropic 10A1; FIV- feline immunodeficiency virus; HIV- human immunodeficiency virus; Int- integrase; MLV- moloney leukemia virus.

luciferase pseudotyped with the VSV-G envelope (Fig. 3-1C). Taken together, these results suggest that the mechanism of resistance is likely not at the receptor level since VSVG facilitates entry via a fusion event mediated by phospholid components of the membrane (Mastromarino et al., 1987) while the 10A1 amphotrophic envelope facilitates entry via one of two receptors, Pit-1 (Glvr-1) or Pit-2 (Ram-1) (Miller and Chen, 1996; Miller and Miller, 1994). Once the block was determined to be post entry, we examined whether or not the block was before integration using an integration defective lentiviral vector encoding EGFP (Fig. 3-3B). Integrase defective lentiviral vectors can complete reverse transcription, and express a transgene at a very low level without having to integrate into the host cell genome. When both V79-4 and 67-1 cells were analyzed by flow cytometry the data indicated that 67-1 cells were refractory to infection between 10 and 25 fold, consistent with what had been observed with an integrase positive retroviral vector encoding EGFP (Fig 3-1A). Through these two assays we concluded that the block to infection in 67-1 cells is post entry, but before integration.

Since we determined that 67-1 was resistant to both MLV and HIV based vectors, we expanded our analysis to another lentiviral vector, FIV. Feline immunodeficiency virus (FIV) is a lentivirus that affects domesticated housecats worldwide, is the causative agent of feline AIDS, and is very similar to HIV (Richards, 2005). When 67-1 and V79-4 cells were challenged with an FIV vector encoding EGFP, the same resistance phenotype was observed (Fig. 3-3C) suggesting that the block in 67-1cells extends to a number of retroviruses.

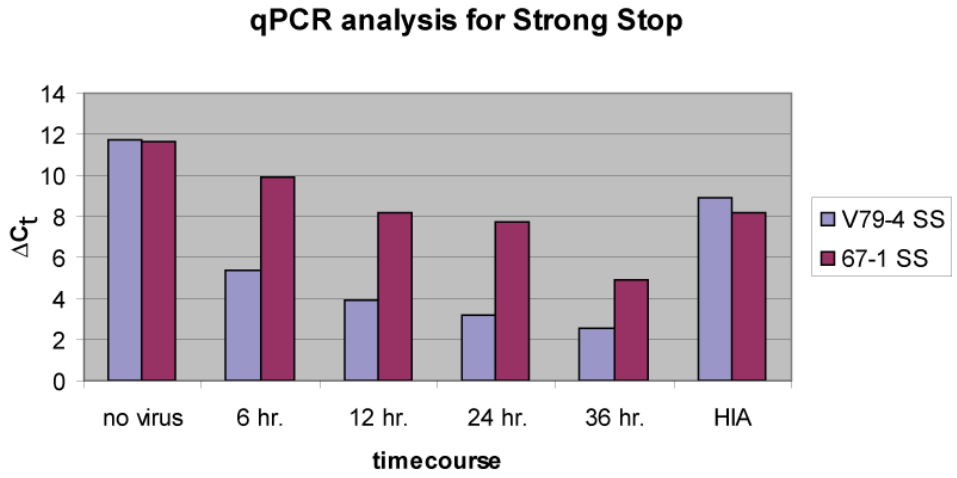
Nearly all of the lentiviral vectors used in this study have utilized the packaging construct ΔNRF and only contain a limited number of HIV accessory proteins: Tat, Rev,

and Vpu (Xu et al., 2001). To determine if the addition of accessory proteins Nef, Vpr, and Vif could restore infectivity, we generated a lentiviral vector encoding EGFP that contained all the accessory proteins.[2] 67-1 was also resistant to a lentiviral vector containing all accessory proteins at a similar level to the lentiviral vectors lacking certain accessory proteins (data not shown). Given that 67-1 is resistant to MLV, resistant to lentiviral vectors based on HIV and FIV, and the presence of all the HIV accessory proteins does not restore infectivity, it is likely that 67-1 is resistant in a pathway that is common to all of these retroviruses.

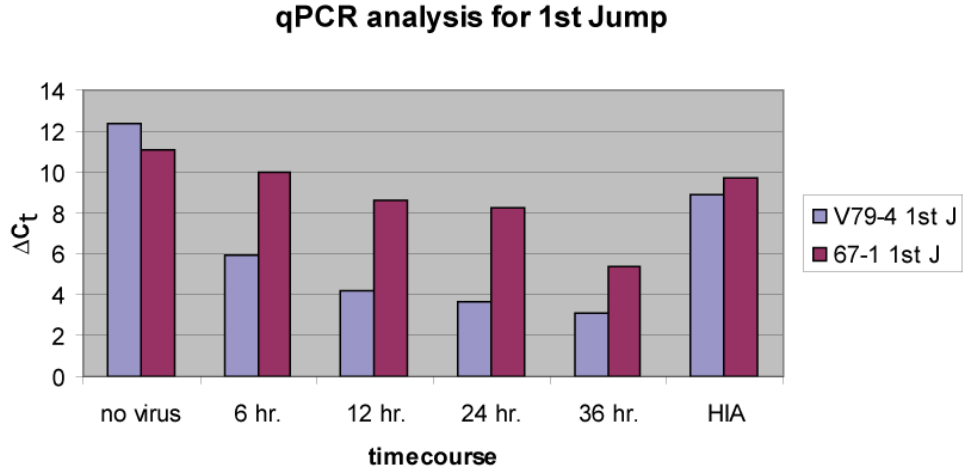
To determine if the block to infection in 67-1 was occurring during the process of reverse transcription, qPCR analysis was conducted to monitor the progression of viral DNA synthesis. Primers were designed to monitor the appearance of reverse transcribed products, specifically strong stop, 1st jump, and full reverse transcribed DNA products (diagrammed in Fig. 2-10A). The data is presented as ΔC_t and is based on amount of PCR product compared to the amount of β-actin present in the same sample where a lower ΔC_T value indicates a greater amount of retroviral product present in the sample (Fig. 3-4). We determined that at every time point, and for every PCR product, the amount of product present in 67-1 cells is less than the parental wildtype V79-4 cells. Based on ΔC_t calculations, we observed an approximate 20 fold reduction in PCR products present in 67-1 cells compared to V79-4 cells, consistent with other assays that have been used to determine fold resistance at a similar m.o.i. (m.o.i.=0.5 for this experiment). Since the 67-1 qPCR analysis showed a decrease in all PCR products, this implied that the block to infection occurs very early after entry, at or before the earliest

[2] Another member of the lab, Patrycja Lech, conducted this experiment.

A


qPCR analysis for Strong Stop
14
12
10
8
6
4
2
0
ΔCt
no virus
6 hr.
12 hr.
24 hr.
36 hr.
HIA
timecourse
V79-4 SS
67-1 SS

B


qPCR analysis for 1st Jump
14
12
10
8
6
4
2
0
ΔCt
no virus
6 hr.
12 hr.
24 hr.
36 hr.
HIA
timecourse
V79-4 1st J
67-1 1st J

C

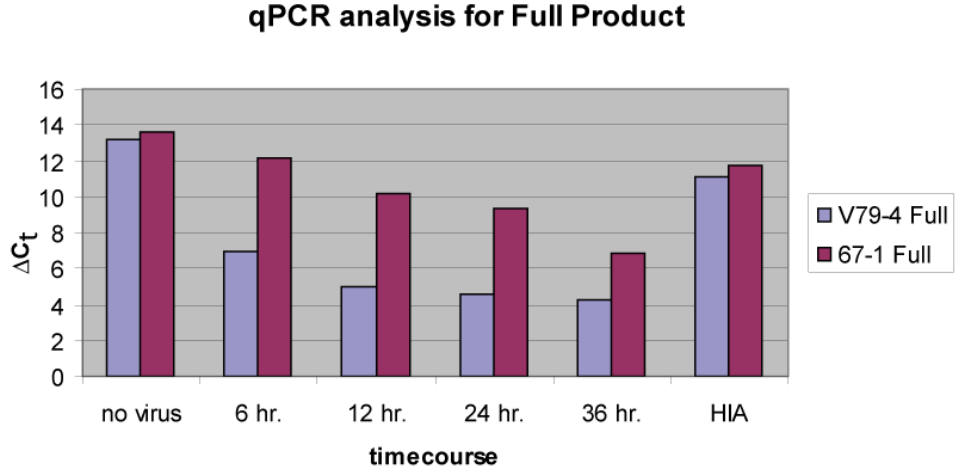

qPCR analysis for Full Product
16
14
12
10
8
6
4
2
0
ΔCt
no virus
6 hr.
12 hr.
24 hr.
36 hr.
HIA
timecourse
V79-4 Full
67-1 Full

Figure 3-4

qPCR analysis to determine if the block occurs during the process of reverse transcription.

qPCR was used to quantify the appearance of reverse transcribed viral DNA. The ΔC_t is based on the amount of PCR product compared to a loading control (β-actin) and the lower ΔC_t value, the greater the amount of PCR product present in the sample. Since 67-1 cells have consistently less PCR product as compared to V79-4 across all stages (SS, 1st J, or Full) and time points, the block to infection must occur after entry but before or at the initiation of reverse transcription. (A) Strong stop qPCR. (B) 1st Jump qPCR. (C) Full- Full/end stage product qPCR. C_t- cycle threshold; Full- full or end stage; HIA- heat inactivate; hr- hours; SS- strong stop; 1st J- first Jump.

step of reverse transcription. Other cell lines have been reported in the literature which appear to be blocked at this step (Gao and Goff, 1999; Naghavi et al., 2005). We concluded based on the qPCR data that the block to infection is after entry but at or just before the initiation of reverse transcription.

Given the stage of the block to infection, we next decided to follow the course of p24, the HIV capsid protein (Gelderblom et al., 1987), after it entered the cell.[3] We infected parental V79-4 cells and resistant 67-1 cells for six hours with a lentiviral vector. The majority of p24 in the wildtype V79-4 cells was seen in the two fractions of the lowest sucrose density (Fig. 3-5A). This is consistent with the interpretation that after cytoplasmic entry the viral core disassociates into its smaller components via the process of uncoating (Bukrinsky et al., 1993b). However in 67-1 mutant cells the majority of the p24 immunoreactivity was apparent in fractions of much higher sucrose density (Fig. 3-5A). The integrity of the light fraction in 67-1 cells was confirmed by the presence of control protein DHFR (dihydrofolate reducatase) in the low density fraction (Fig. 3-5A). The p24 immunoreactivity in the higher density fractions in 67-1, led us to hypothesize that the p24 may be in a complex undergoing degradation given the broad signal in the immunoblot. To test this notion we re-probed the blot with an antibody to the 20S subunit of the proteasome (Fig. 3-5A). The 20S subunit of the proteasome was located in fractions 7 and 8 in both V79-4 and 67-1 cells, the same fraction that contained p24 immunoreactivity in 67-1 cells. These results led us to a model, given the presence of both p24 and the 20S subunit of the proteasome in the same fraction, that 67-1 may have

[3] Another member of the lab, Jeff Schreifels, conducted this experiment.

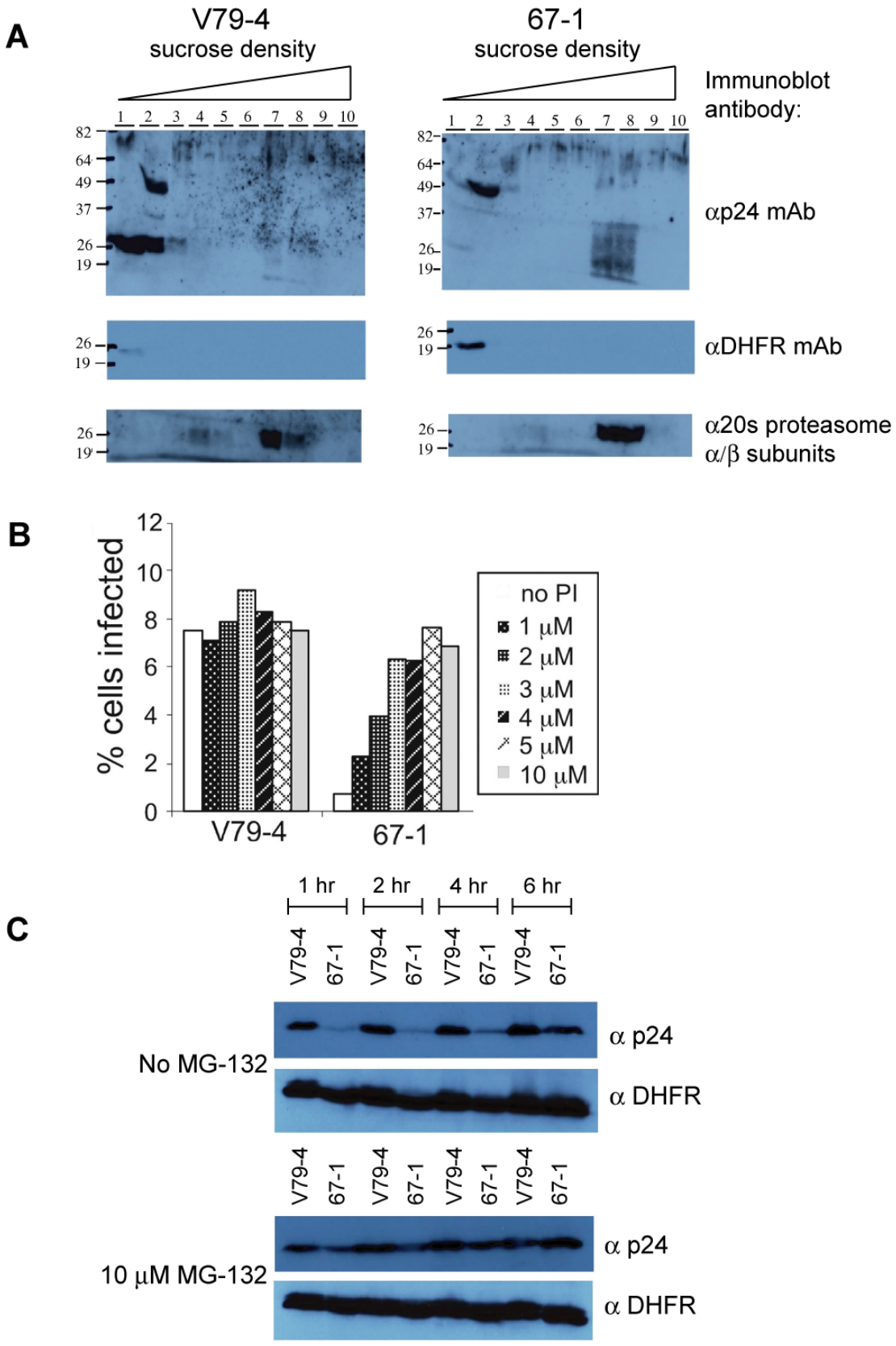

A
V79-4
sucrose density
67-1
sucrose density
Immunoblot antibody:
1 2 3 4 5 6 7 8 9 10
82
64
49
37
26
19
αp24 mAb
αDHFR mAb
α20s proteasome α/β subunits
B
% cells infected
12
10
8
6
4
2
0
V79-4
67-1
no PI
1 μM
2 μM
3 μM
4 μM
5 μM
10 μM
C
1 hr
2 hr
4 hr
6 hr
V79-4
67-1
No MG-132
α p24
α DHFR
10 μM MG-132

Figure 3-5

Pinpointing the block to infection.

(A) After six hours of infection with a lentiviral vector, the distribution of HIV-1 capsid (p24) in wildtype V79-4 and mutant 67-1 cells was examined via protein fractionation on a 20%-70% sucrose gradient. Fractions were probed with either mouse anti-p24 (αp24 mAb), mouse anti-dihydrofolate reductase Ab (αDHFR mAb), or rabbit anti-20S proteasome α/β subunits. (B) V79-4 and 67-1 cells were preincubated with the proteasome inhibitor (PI) MG-132 at various concentrations, infected with a lentiviral vector trasducing EGFP, and analyzed by flow cytometry for EGFP positive cells. (C) V79-4 and 67-1 cells were preincubated with or without 10 μg MG-132 for four hours and challenged with a lentiviral vector. Cell lysates were prepared at specific time points post infection (1, 2, 4, and 6 hours), and p24 levels were detected by western blot. As a loading control, the immunoblots were reprobed with αDHFR. The data presented in Figure 3-5A was generated by Jeff Schreifels, another member of the lab. The data presented in Figure 3-5B and Figure 3-5C were generated by Patrycja Lech, another member of the lab. αDHFR mAb- mouse anti-dihydrofolate reductase Ab; αp24 mAb- mouse anti-p24; PI- proteasome inhibitor.

a defect in uncoating that leads to its resistance phenotype and that this resistance phenotype involves the activity of the proteasome.

Rescuing the block to infection

A prediction of this model is that infectivity may be restored in 67-1cells by treating the cells with the proteasome inhibitor MG-132. We preincubated V79-4 and 67-1 cells with varying concentrations of MG-132 (0-10 μM) and then infected with a lentiviral vector encoding EGFP (Fig. 3-5B).[4] Incubation with MG-132 caused both V79- 4 cells and 67-1 cells to have greater susceptibility to lentiviral vector infection, but the level of susceptibility varied. In V79-4 cells, the greatest increase occurred at a concentration of 3 μM and this increase was only 1.2 fold. However, in 67-1 cells the greatest increase in infectivity occurred at a concentration of 5 μM and this increase resulted in levels of infection to near wildtype levels. Once we determined that restoration of infectivity could be established by suppressing protease function, we next determined if restoration of infectivity correlated with p24 stabilization.

V79-4 and 67-1 cells were preincubated with MG-132, or untreated for a control, and then infected with a lentiviral vector in the presence of MG-132. The cells were collected 1, 2, and 6 hours after infection and the cell extracts immunoblotted to determine the relative levels of p24 in the cell lysates (Fig 3-5C).[5] In the absence of MG-132, even at the earliest time point, p24 was detectable in V79-4 cells, but in 67-1 cells, p24 levels were reduced across all time points relative to V79-4 cells. However, when the cells were infected in the presence of MG-132, 67-1 showed a marked increase in p24

[4] Another member of the lab, Patrycja Lech, conducted this experiment.
[5] Another member of the lab, Patrycja Lech, conducted this experiment.

levels and the levels were comparable to those observed in wildtype V79-4 cells. We concluded that suppression of proteasome activity with MG-132 in 67-1 cells restores infectivity and stabilizes p24 to wildtype levels.

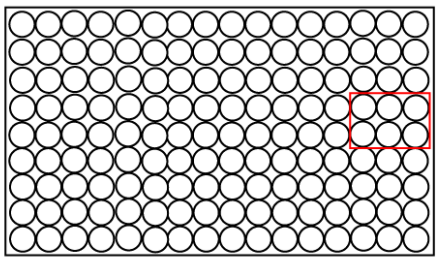
Identifying MRI

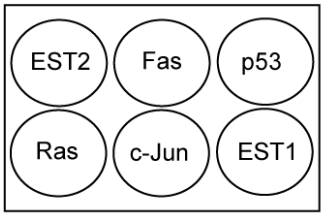
Once 67-1 had been characterized and shown to be resistant to retrovirally mediated transduction, we initiated a gain of function rescue screen. Since the phenotype was one of partial resistance, and not of complete resistance, a standard gene complementation screen, i.e. introduction of an expression cDNA library in bulk culture was not feasible. For example, to screen a total of 10^6 novel cDNA sequences, the background level of cells that would be infectable even without the cDNA would be 10^5-10^4 clones (since 67-1 is minimally 10 fold refractory to infection). Hence to identify the gene conferring resistance we utilized a novel complementation assay called GFAcT (Genome Functionalization through Arrayed cDNA Transduction), the basic outline of this methodology is illustrated in Fig. 3-6.

GFAcT has been utilized previously to identify novel interactors of AP-1 (Chanda et al., 2003) and CREB (Conkright et al., 2003) via cotransfection of a cDNA expression library along with a construct expressing luciferase under control of either the AP-1 or CREB responsive promoter. GFAcT has also been used to identify novel activators of Kaposi Sarcoma-Associated Herpesvirus by transfecting a cDNA expression library into cells harboring a latent form of the virus (Yu et al., 2007). Using GFAcT our collaborators at the Genomics Institute of the Novartis Foundation (GNF) analyzed over 11,000 unique human and mouse cDNAs for complementation in a transient

GFAcT: Genome Functionalization through Arrayed cDNA Transduction

An automated system plates cDNAs onto 384 well plates





Each well contains an expression construct for a protein of known or unknown function.

Add Fugene to each well. Retro(transfect) V79-4 and 67-1 cells in parallel.

Using this method, greater than 11,000 unique human and mouse cDNAs were tested for complementation with 67-1.

24 hours later challenge each well with a lentiviral vector encoding luciferase.

48 hours later measure the luciferase activity in to identify genes increasing luciferase activity in 67-1 cells compared to the controls.

Figure 3-6

GFAcT protocol. This protocol was used to identify genes that would complement the mutation in 67-1.

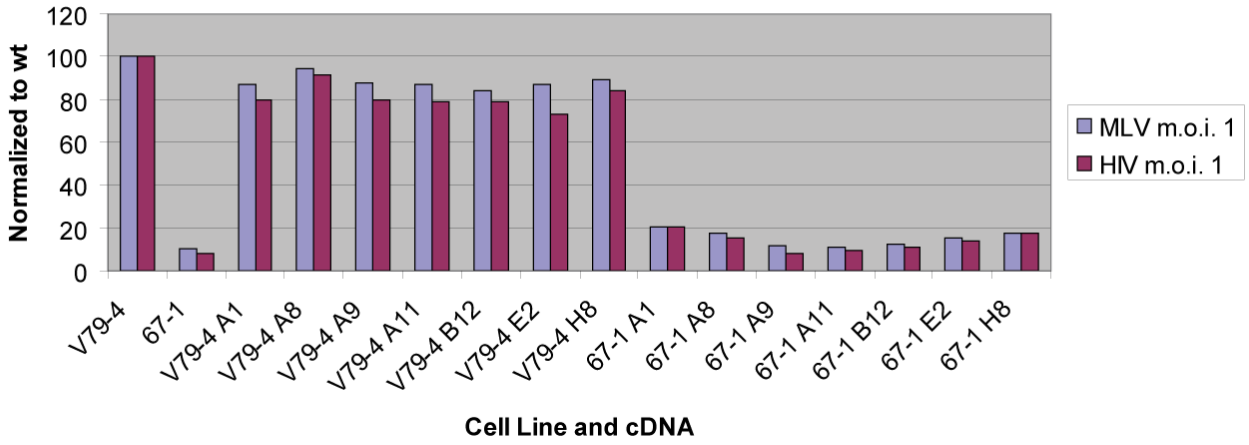
overexpressed system. Since the transfection efficiency of the cDNA expression clone is not 100% we expected that this method would allow us to partially rescue our recessive phenotype.

GFAcT identified cDNAs that increased the level of luciferase expression in mutant 67-1 cells as compared to wildtype V79-4 cells. Briefly, 67-1 or V79-4 cells were transfected with 11,000 cDNA expression clones in a 384 well format and subsequently infected with a lentiviral vector transducing the luciferase gene. Forty-eight hours later, luciferase expression from each well was quantified. All the manipulations were automated and data was collected in duplicate for each cDNA during this high throughput screen. The mean luciferase activity was calculated by summing all experimental wells. cDNA clones from wells in which luciferase activity was above one standard deviation from the mean were taken for further analysis. A 1:4 ratio of V79-4 and 67-1 cells, which acted a control, with the assumption that this would mimic a 25% transfection efficiency of a correcting clone, passed this test (See Materials and Methods). Next, V79-4 and 67-1 data sets were compared to identify cDNAs that resulted in an increase in luciferase activity more than one standard deviation above the mean for both V79-4 and 67-1. These cDNAs were eliminated from further analysis because they likely did not complement the mutation and were artificially increasing luciferase readings by either activating the promoter or increasing cell proliferation. GFAcT was repeated using the cDNA clones which increased luciferase expression in 67-1 cells but not in V79-4 cells. For the repeat experiment, cells were separately infected with CSII-luciferase retroviral vector and transfected with the CSII-luciferase plasmid vector. At this point, cDNAs that increased luciferase activity upon both transfection and infection were eliminated from

future study as these did not uniquely rescue the infection resistance phenotype. This filtering scheme resulted in eight cDNAs that increased luciferase readings at a significant level in 67-1 cells after infection, but did not increase luciferase levels in V79-4 cells.

To examine the ability of these cDNAs to rescue the mutant phenotype, they were singly transfected into V79-4 and 67-1 cells along with a selectable marker for puromycin resistance and subsequently selected with puromycin. These puromycin resistant populations, each expressing a single cDNA heterogeneously, were infected with a lentiviral vector encoding EGFP and analyzed by flow cytometry (Fig. 3-7A). The heterogeneous population of cells expressing A1 resulted in the greatest level of rescue in 67-1 cells. We determined that transfection of the A1 cDNA expression construct into 67-1 cells resulted in a level of infectivity that was higher than the original 67-1 line by approximately two fold; however it still was not as infectable as the parental V79-4, indicating a partial recovery of infectivity. We hypothesized that the 67-1 cells selected with puromycin might have varied expression of the cDNA so we isolated subclones from the pool. The 67-1 A1 transfected pool was plated out at a very low density and individual subclones, with homogenous expression of the A1 cDNA, were examined further by flow cytometry. One clone, #5, showed a much greater level of infectivity as compared to the originally resistant 67-1 cells (Fig. 3-7B). Complemented clone #5 showed a level of infectivity that was between 70-90% of the parental V79-4, and a greater level of rescue of infectivity was observed for lentiviral vectors compared to MLV retroviral vectors. The cDNA found in A1 (BC000168, Gene Expression Omnibus Database) is a previously uncharacterized gene that we renamed MRI (**M**odulator of

A


FACS Analysis of pooled cDNA stable Cell Lines
Normalized to wt
120
100
80
60
40
20
0
V79-4
67-1
V79-4 A1
V79-4 A8
V79-4 A9
V79-4 A11
V79-4 B12
V79-4 E2
V79-4 H8
67-1 A1
67-1 A8
67-1 A9
67-1 A11
67-1 B12
67-1 E2
67-1 H8
Cell Line and cDNA
MLV m.o.i. 1
HIV m.o.i. 1

B

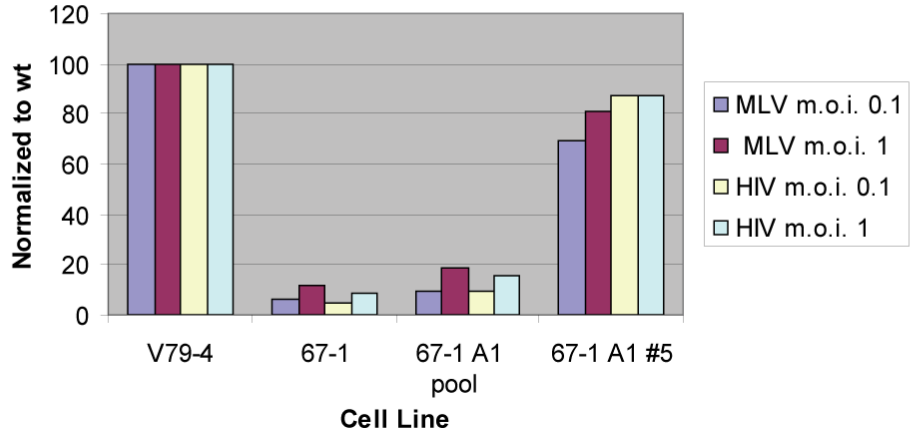

FACS Analysis of MLV and Lentiviral Infections
Normalized to wt
120
100
80
60
40
20
0
V79-4
67-1
67-1 A1 pool
67-1 A1 #5
Cell Line
MLV m.o.i. 0.1
MLV m.o.i. 1
HIV m.o.i. 0.1
HIV m.o.i. 1

Figure 3-7

The resistance phenotype in 67-1 can be rescued.

(A) V79-4 and 67-1 cells were cotransfected with a candidate cDNA and a plasmid encoding the gene for puromycin resistance. The puromycin resistant clones were pooled, infected with a lentiviral vector encoding EGFP, and analyzed by flow cytometry. Data is presented relative to V79-4 (wildtype levels). Clone A1 gave the greatest level of partial rescue and was analyzed further. (B) Individual puromycin resistant clones were isolated and tested for infection against MLV and lentiviral vectors encoding EGFP. Data are presented relative to V79-4 (wildtype levels). One clone, #5, had rates of infection that recovered to nearly wildtype levels. FACS- flow assisted cell sorting; m.o.i.- multiplicity of infection; wt- wildtype.

Retroviral **I**nfection), since it was able to modulate the infectivity of a previously refractory cell line. V79-4 cells were also transfected with MRI cDNA and the pooled population and individual subclones showed no difference in the level of infectivity as compared to the wild-type V79-4 cells (Fig. 3-7A, individual subclone data not shown). Based on this data we concluded that BC000168 (MRI) rescued the resistance phenotype in 67-1 cells.

Expression profile and localization

cDNA A1, what we have termed MRI, codes for human protein MGC5242 (Mammalian Gene Consortium Database). MRI is a 157 amino acid protein with a size of 22kDa with no known functions or domains. MRI is conserved among mammals with an average of 56% identity and 62% similarity to human MRI. Additionally, an avian version of MRI has 34% identity and 51% similarity to the human version (Fig. 3-8). Although MRI has no known function or domains, gene chip analysis has indicated it is ubiquitously expressed. The gene expression profile can be viewed at http://symatlas.gnf.org using the search term BC000168. According to this expression profile, MRI is expressed in many human tissue types, including T cells, a target cell type for HIV-1 infection.

To examine the localization of MRI, we generated an expression vector capable of transducing a 3x N-terminally Flag tagged MRI (CSII-3x Flag MRI). We transfected cell lines with this plasmid to examine the localization of MRI. V79-4, 67-1, MRC-5, A-498, and 293T cells were transfected, fixed, and the localization of the tagged MRI-1 protein determined by α-FLAG antibody immunofluorescence (Fig. 3-9A-E). The

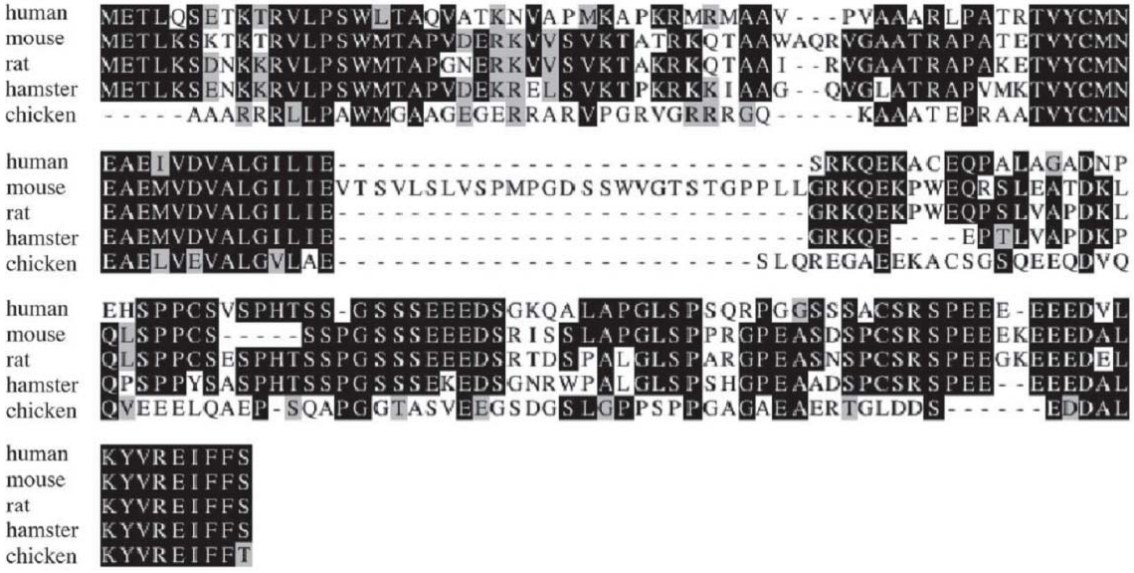

human METLQSETKTRVLPSWLTAQVATKNVAPMKAPKRMRMAAV---PVAAARLPATRTVYCMN
mouse METLKSKTKTRVLPSWMTAPVDERKVVSVKTATRKQTAAWAQRVGAATRAPATETVYCMN
rat METLKSDNKKRVLPSWMTAPGNERKVVSVKTAKRKQTAAI--RVGAATRAPAKETVYCMN
hamster METLKSENKKRVLPSWMTAPVDEKRELSVKTPKRKKIAAG--QVGLATRAPVMKTVYCMN
chicken -----AAARRRLLPAWMGAAGEGERRARVPGRVGRRRGQ-----KAAATEPRAATVYCMN
human EAEIVDVALGILIE----------SRKQEKACEQPALAGADNP
mouse EAEMVDVALGILIEVTSVLSLVSPMPGDSSWVGTSTGPPLLGRKQEKPWEQRSLEATDKL
rat EAEMVDVALGILIE----------GRKQEKPWEQPSLVAPDKL
hamster EAEMVDVALGILIE----------GRKQE----EPTLVAPDKP
chicken EAELVEVALGVLAE----------SLQREGAEEKACSGSQEEQDVQ
human EHSPPCSVSPHTSS-GSSSEEEDSGKQALAPGLSPSQRPGGSSSACSRSPEEE-EEEDVL
mouse QLSPPCS-----SSPGSSSEEEDSRISSLAPGLSPPRGPEASDSPCSRSPEEEKEEEDAL
rat QLSPPCSESPHTSSPGSSSEEEDSRTDSPALGLSPARGPEASNSPCSRSPEEGKEEEDEL
hamster QPSPPYSASPHTSSPGSSSEKEDSGNRWPALGLSPSHGPEAADSPCSRSPEE--EEEDAL
chicken QVEEELQAEP-SQAPGGTASVEEGSDGSLGPPSPPGAGAEAERTGLDDS------EDDAL
human KYVREIFFS
mouse KYVREIFFS
rat KYVREIFFS
hamster KYVREIFFS
chicken KYVREIFFT

Figure 3-8

Sequence Alignment.

Protein sequence comparison of MRI between human, mouse, rat, hamster, and chicken.

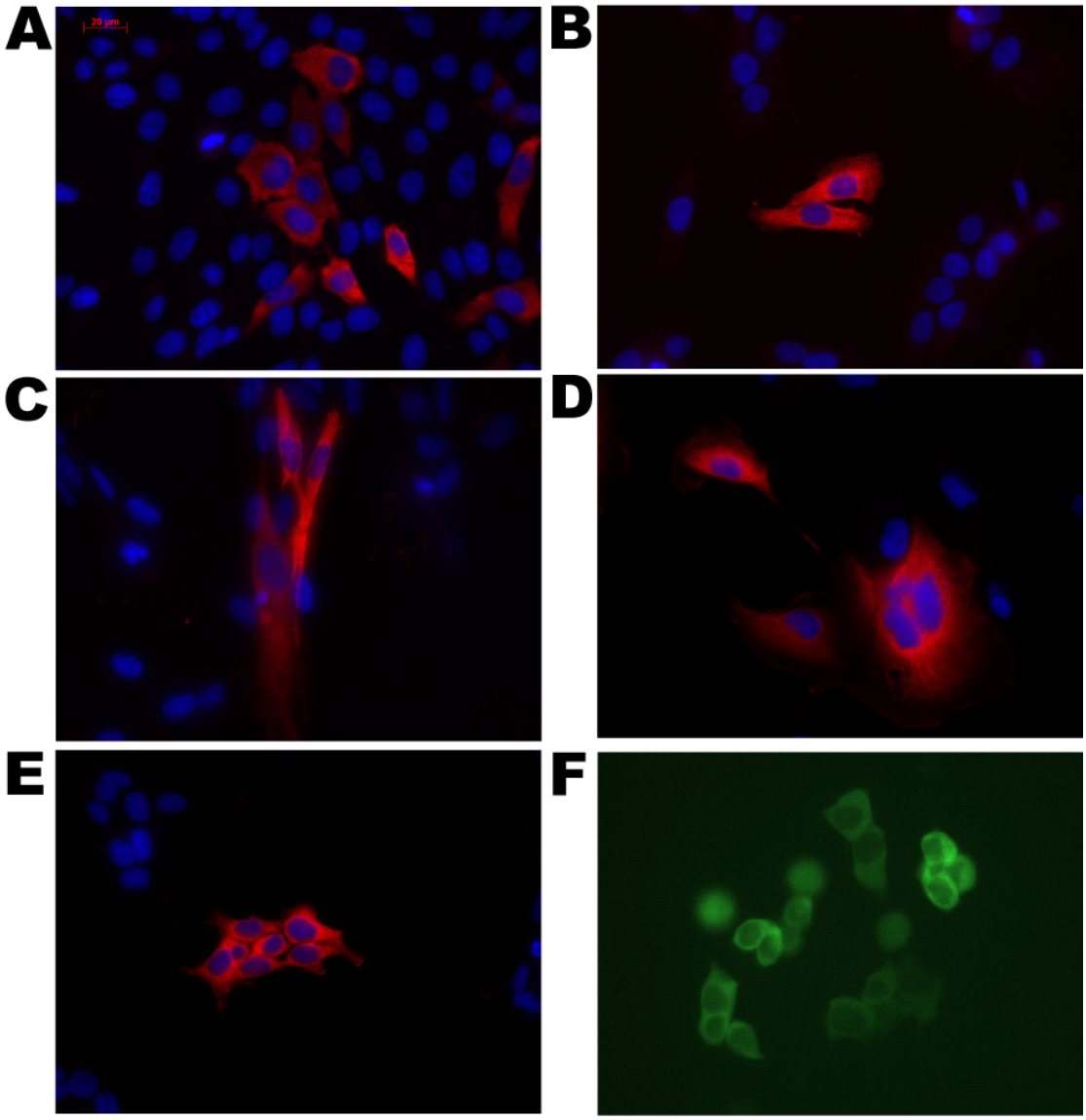

A
20 µm
B
C
D
E
F

Figure 3-9

Localization of tagged MRI in various cell lines.

Panels A-E contain images of fixed cells where red fluorescence marks flag-tagged MRI and blue fluorescence indicates DAPI-stained nuclei. Figure F is a live image of an N-terminally fused GFP-MRI fusion protein. (A) Wildtype hamster V79-4. (B) Resistant hamster 67-1. (C) Mouse MRC-5. (D) Human A-498. (E) Human 293T. (F) Human HeLa.

localization among all the cell lines was very consistent. All cell lines showed cytoplasmic localization of MRI and very little protein within nuclei. The block to infection in 67-1 is post entry but before integration, and the events during this portion of the HIV lifecycle all occur in the cytoplasm. Therefore, the localization of MRI in the cytoplasm coincides with where MRI may be functioning to overcome the block to infection in 67-1 cells. The localization of MRI in both V79-4 and 67-1 cells was the same, indicating that the mutation in 67-1 cells which confers resistance does not change the localization of the tagged MRI (Fig. 3-9A-B).

We also generated an expression construct encoding an N-terminally fused EGFP-MRI protein and transfected this fusion construct into HeLa cells (Figure 3-9F). MRI localization in live cells was consistent with localization determined by immunofluorescence, localizing to the cytoplasm and very little GFP within the nucleus. This localization of the EGFP-MRI did not change upon infection with a lentiviral vector (data not shown).

Following the localization study, we wanted to determine if there was a mutation in 67-1 MRI and determine if this mutation was causing the resistance phenotype. To do this we first needed to characterize wildtype hamster MRI. Total RNA was harvested from V79-4 cells and used to generate cDNA. The cDNA was then subjected to PCR using 5' and 3' primers derived from rodent sequences homologous to human MRI. This PCR resulted in the isolation and sequencing of the previously undetermined wildtype hamster MRI cDNA (deposited in Genbank as DQ899952). We next amplified the MRI cDNA from 67-1 total RNA. The MRI cDNA isolated from 67-1 cells did not contain any mutations in the transcript when compared to the sequence of MRI from V79-4 cells.

We concluded that the 67-1 MRI was identical to the wildtype V79-4 MRI. This result was verified from two independent cDNA reactions from two independent total mRNA preparations. We next quantified MRI transcript levels using qPCR to examine the possibility that the resistance phenotype was due to altered expression levels. qPCR of MRI levels normalized to β-actin determined there was no difference in the levels of MRI transcript between V79-4 and 67-1 cells (Fig. 3-10). We concluded from this data that MRI must be acting as an overexpression suppressor in 67-1, since excess MRI in the mutated 67-1 cells was restoring infectivity to the previously resistant cell line.

Knockdown of MRI results in a resistance phenotype

Since we determined that MRI was functioning as an overexpression suppressor in 67-1, we next wanted to discern if reduction of endogenous MRI could recapitulate the resistance phenotype seen in 67-1 cells. To do this we first developed shRNA (small hairpin RNA) sequences (Rubinson et al., 2003) that targeted human MRI and utilized these sequences to knockdown the expression of an epitope tagged MRI in a transient system.[6] We initially developed three shRNA sequences (sh623, sh760, and sh353) that targeted MRI but only two (sh623 and sh353) efficiently decreased the expression of a Flag tagged MRI in 293T cells (Fig. 3-11A). To ensure efficient transfections and to serve as a loading control, the cells were cotransfected with a plasmid encoding EGFP and the blotting was repeated using an anti-EGFP antibody (αGFP). sh623 and sh353 were used for further analysis along with a positive control (shFF that targets the firefly luciferase gene) and a negative control (shScram, a scrambled shRNA sequence).

[6] Another member of the lab, Bryan Nikolai, conducted this experiment.

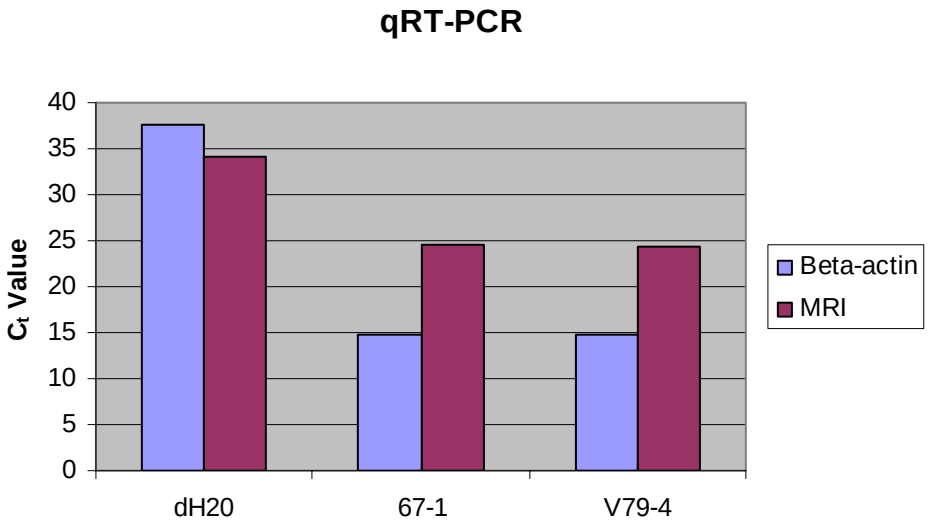
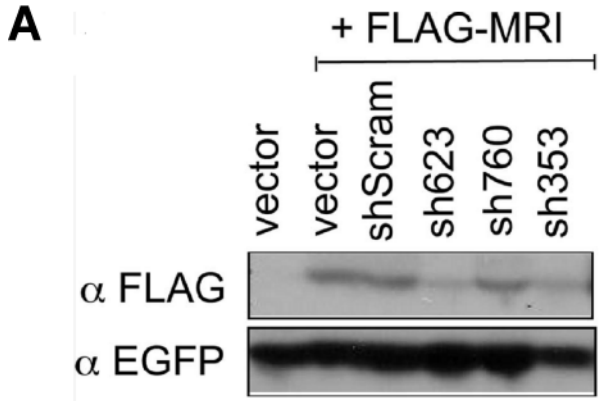

qRT-PCR
40
35
30
25
20
15
10
5
0
Ct Value
dH20
67-1
V79-4
Beta-actin
MRI

Figure 3-10

The amount of MRI transcript present in V79-4 and 67-1 does not differ.

qRT-PCR of MRI mRNA and β-actin in both V79-4 and 67-1 cells indicate no discernable difference between the cell lines. qPCR was conducted with water as a negative control.


A
+ FLAG-MRI
vector
vector
shScram
sh623
sh760
sh353
α FLAG
α EGFP

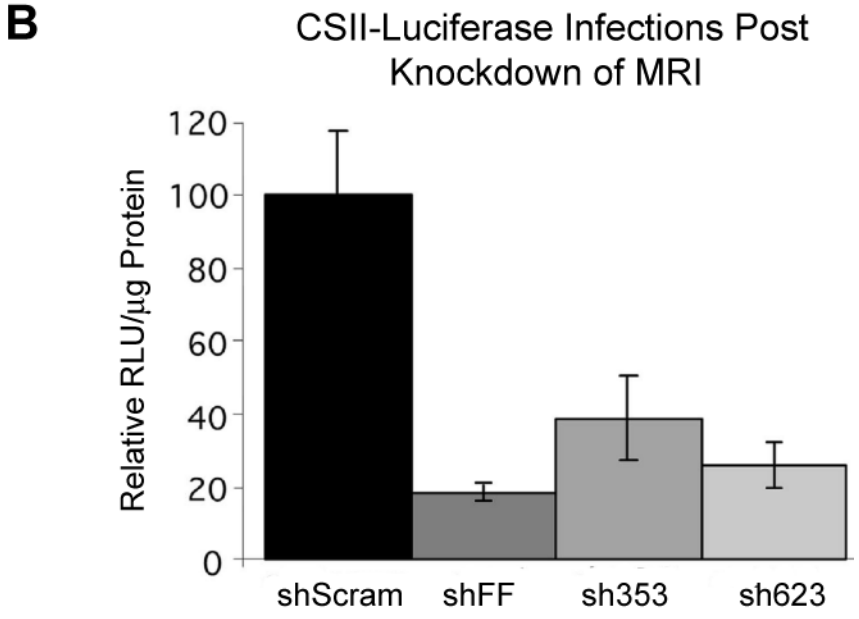

B
CSII-Luciferase Infections Post Knockdown of MRI
Relative RLU/μg Protein
120
100
80
60
40
20
0
shScram
shFF
sh353
sh623

Figure 3-11

Knockdown of MRI results in a resistance phenotype.

(A) Three different shRNA (small hairpin RNA) constructs were tested for efficacy of knockdown of MRI levels in a transient system. An expression construct for Flag-MRI and various shRNA expression vectors were cotransfected into 293T cells and MRI protein expression was detected 48 hours later by western blot with anti-Flag Ab (αFlag). An immunoblot probed with anti-EGFP (αEGFP) served as a loading control, since an expression vector for EGFP was co-transfected with the MRI plasmid. (B) Human U87 cells expressing either shScram, shFF, sh353, or sh623 were challenged with a lentiviral vector encoding luciferase and 48 hours later luciferase readings were conducted. The data are represented as relative to the shScram luciferase reading which served as the negative control. The data presented in Figure 3-11A were generated by Bryan Nikolai, another member of the lab. The data presented in Figure 3-11B were generated by Patrycja Lech, another member of the lab. MRI- modulator of retroviral infection; RLU- relative light units; shRNA- small hairpin RNA; sh353- shRNA targeting MRI; sh623- shRNA targeting MRI; sh760- shRNA targeting MRI; shFF- shRNA targeting firefly luciferase; shScram- a scrambled shRNA.

sh623, sh353, shFF, and shScram were placed into an MLV based vector and used to infect U87 cells.[7] U87 cells were utilized because they have been previously used to monitor the progression of HIV infection (Hatse et al., 2007; Reeves et al., 1999). After infection with the shRNA constructs, the U87 cells were passaged and challenged with a lentiviral vector encoding luciferase (Fig. 3-11B). As expected, the shFF effectively reduced luciferase expression as compared to the scrambled sequence (18% when normalized to the negative control shScram). Additionally both MRI targeting sequences also had an effect on infection by reducing the luciferase levels to 39% (sh353) and 26% (sh623) when normalized to shScram. We repeated this assay in another human cell line, HOS, and a different reporter, CSII-EF-dsRed, and observed similar results (data not shown). Based on the shRNA data we concluded that knockdown of endogenous MRI protein can lead to a phenotype similar to that observed in 67-1, a resistance to lentivirally mediated transduction.

Discussion

In this study, we have isolated and characterized a mutant cell line, 67-1, that is refractory to retroviral infection as compared to the wildtype V79-4 cell line. The resistance phenotype is caused by a recessive mutation, cannot be easily saturated, and cannot be overcome with alternative promoters. By using different envelopes, integrase defective viruses, and qPCR we determined that the block to infection is post entry and either at or just before the initiation of reverse transcription. By investigating viral fate, we determined that 67-1 cells destabilize p24, a viral core protein, and this destabilization can be overcome by treating 67-1 cells with a proteasome inhibitor. Furthermore,

[7] Another member of the lab, Patrycja Lech, conducted this experiment.

treatment with a proteasome inhibitor results in a rescue of infectivity in 67-1 cells to near wildtype levels. We restored infectivity to 67-1 cells by overexpressing a novel gene, MRI, isolated using a high throughput complementation cloning approach. We also determined that endogenous 67-1 MRI cDNA (and hence mRNA) sequence did not harbor a mutation and there is no discernable difference in mRNA expression levels of MRI between 67-1 and V79-4 cells. The data indicate that MRI is an overexpression suppressor of the mutation that confers the resistance phenotype in 67-1 cells.

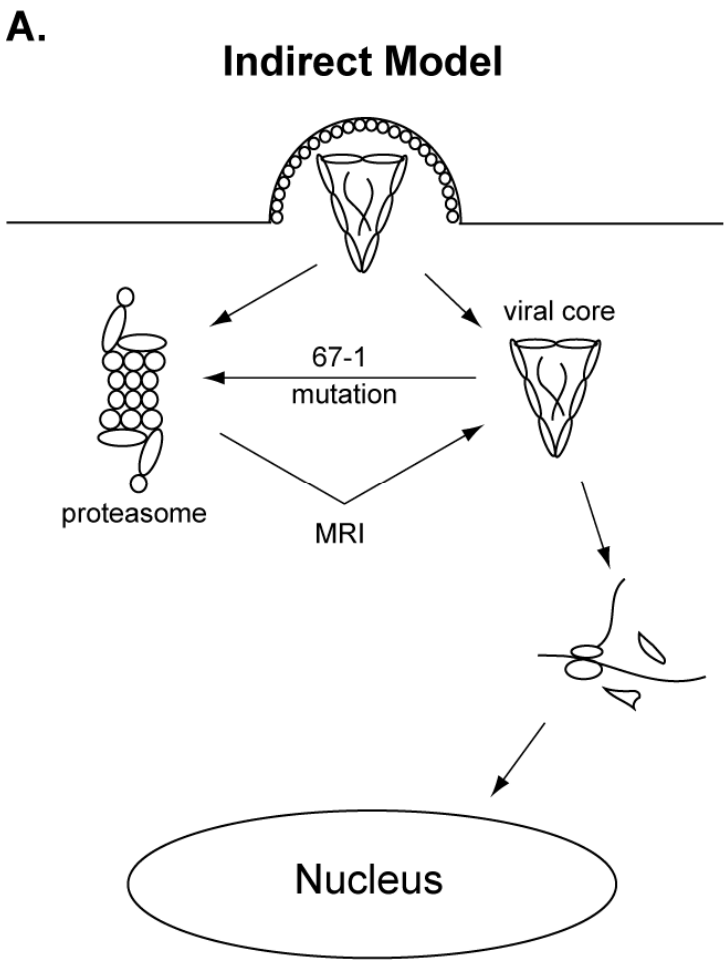
A role for the proteasome in HIV infections

It is clear from these data that 67-1 cells are resistant to retrovirally mediated transduction post entry but before or at the initiation of reverse transcription and that overexpression of MRI can overcome this resistance. It is at this stage that viral uncoating occurs. Uncoating is the process by which the cytoplasmically localized viral core composed primarily of capsid proteins, including p24, disassociates and allows for the initiation of the reverse transcription and preintegration complex formation (Nisole and Saib, 2004). Uncoating is a poorly understood area of the retrovirus life cycle, however utilizing techniques that allow researchers to track p24 through density based cellular fractionations has shed new light on the process. Previous studies examining HIV-1 uncoating (Fassati and Goff, 2001) and now our study have shown that p24 is generally found in the lighter fractions. However, our study has shown that p24 migrates to a much heavier fraction, a fraction that coincides with the proteasome, in 67-1 cells resistant to retrovirally mediated transduction. The role of the proteasome in retroviral infections is not unprecedented. The innate retroviral defenses, APOBEC3G and Trim5α, are both targeted for degradation by the proteasome. It has been proposed that

APOBEC3G degradation does not require that it become polyubiquitylated, but instead may require that the HIV accessory protein Vif undergo polyubiquitination (Dang et al., 2008). It has been shown that Trim5α is rapidly degraded by the proteasome when exposed to restriction-sensitive retrovirus, but not when exposed to unrestricted virus (i.e. when rhesus Trim5α was exposed to HIV viral cores, there was a rapid degradation of Trim5α, however when human Trim5α was exposed to HIV viral cores there was little degradation) (Rold and Aiken, 2008). Additional studies have determined that if proteasome activity is suppressed it can both enhance infectivity (Wei et al., 2005) and reduce budding (Ott et al., 2003). Our results indicate that inhibition of the proteasome does increase retroviral infections and that the proteasome plays a role early in the retroviral cycle.

Two models for HIV infection

We propose two models based on the data presented in this study that incorporate the proteasome in the process of uncoating. The first model gives the proteasome an indirect role in the retroviral lifecycle (Fig. 3-12A). The viral core enters the cytoplasm and the viral core is either recognized and degraded by the proteasome or it uncoats and completes the early phase of infection. In this model, the mutation in 67-1 slows down the uncoating process and consequently a larger percentage of entering viral cores are degraded by the proteasome. This model explains our observation that even inhibition of proteasome function in wildtype V79-4 cells results in a slight increase in infectivity (Fig. 3-5B) because the few random cores that would have been degraded are also protected. The second model gives the proteasome a direct role in the retroviral lifecycle (Fig. 3-12B). Here, we propose that the viral core enters the cytoplasm and specifically interacts


A.
Indirect Model
viral core
67-1
mutation
proteasome
MRI
Nucleus

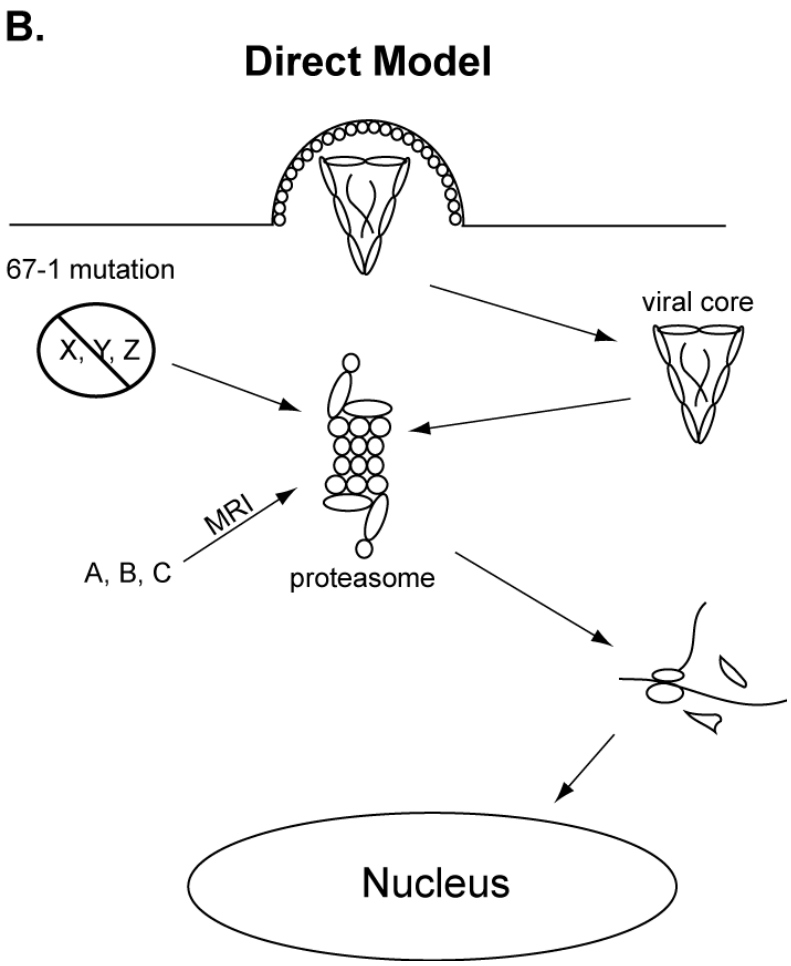

B.
Direct Model
67-1 mutation
X, Y, Z
viral core
MRI
A, B, C
proteasome
Nucleus

Figure 3-12

Models.

(A) Indirect Model. Generally, viral cores enter the cytoplasm and undergo uncoating while in transit to the nucleus. However, viral cores may be shuttled to the proteasome for degradation. The mutation in 67-1 retards the process of viral uncoating, thereby causing greater shuttling of the viral core to the proteasome. Overexpression of MRI may overcome the mutation in 67-1 by shuttling the viral core away from the proteasome. Since MRI plays a role in keeping the viral core away from proteasome, knockdown of endogenous MRI results in a recapitulation of the resistance phenotype seen in 67-1 cells. (B) Direct Model. Generally, viral cores enter the cytoplasm and directly interact with the proteasome to aid in uncoating. At this point, the virus recruits host cell factors (X, Y, Z) that protect it from complete degradation by the proteasome, but on rare occasions the viral core will be completely degraded. The mutation in 67-1 either prevents the recruitment of these protective host cell proteins or the mutation is in one of the protective host cell proteins. Overexpression of MRI results in either excessive recruitment of other protective host cell proteins (A, B, C) or overexpression of a specific protective host cell protein, both of which could compensate for the loss of X, Y, or Z. Knockdown of endogenous levels of MRI results in either lowered levels of recruited protective host proteins (X, Y, Z) or a reduction in a specific protective protein (A, B, C), both of which would recapitulate the resistance phenotype seen in 67-1 cells.

with the proteasome to facilitate uncoating. At this point, the virus recruits host cell proteins to protect it from complete degradation by the proteasome, but a few cores are still degraded. However, the mutation in 67-1 either prevents the virus from recruiting these protective host proteins or 67-1 is mutated in a protective host protein. This model also explains our observation that wildtype cells have increased infectivity when we inhibit proteasome function because the cores that are normally degraded have increased protection. Both of these models fit the data presented in this study, and further analysis will be necessary to identify the correct one.

The two proposed models also take into account our observation that knockdown of endogenous MRI results in a resistance phenotype (Fig. 3-11) and our hypothesis that MRI acts as an overexpression suppressor of the mutation in 67-1 cells. In the indirect model (Fig. 3-12A) the overexpression suppressor MRI facilitates proper core uncoating thereby overcoming the mutation found in 67-1 and if endogenous MRI is suppressed that slows down the process of uncoating, recapitulating the phenotype of 67-1 cells. In the direct model (Fig. 3-12B) the overexpression suppressor MRI excessively recruits host cell proteins to protect the viral core when the 67-1 mutation is present. Under the direct model the knockdown of endogenous MRI in normal cells results in a recapitulation of the 67-1 phenotype because MRI cannot recruit the host cell proteins necessary to protect the viral core. Hence, knockdown of endogenous MRI recapitulates the phenotype observed in the mutant 67-1 cells, it can fit into either of the proposed models.

Host cell proteins: implications for treatment

The isolation and characterization of MRI is important because it identifies another host cell protein involved in retrovirally mediated transduction. Identification of host cell proteins is essential to further the understanding of retrovirally mediated transduction and gene therapy. Furthermore, any newly identified host proteins can aid in the search for additional HIV therapies. Current therapies predominantly involve the targeting of HIV specific activities or proteins. However, given the high rate of mutations associated with HIV reverse transcriptase (1 in 7000 nucleotides) (Ji and Loeb, 1992) and the high viral turnover (up to 10^9 viral particles per day) (Drosopoulos et al., 1998), HIV can become resistant to the drugs. Current therapies are a two edged sword; while people do live longer under Highly Active Antiretroviral Therapy (HAART) they are also developing multi-drug resistant strains of HIV, transmitting multi-drug resistant strains of HIV, and these resistant strains are very difficult to treat (Temesgen et al., 2006). A novel approach to HIV therapy would be to target a non-essential host protein that HIV requires to complete its life cycle. Targeting host proteins as opposed to the HIV proteins could lead to therapies where the high mutation rate of HIV cannot overcome the drug blockade. Although significantly more work is necessary to determine if MRI can be targeted for new therapy, the possibility exists that a host cell protein could become a potential anti-retroviral target. Moreover, this host cell protein based HIV therapy could become the new standard for HIV therapy.

CHAPTER 4

Conclusions

The goal of my project was to identify a non-essential host cell protein required for retroviral infection, ultimately to be used as a possible target for HIV drug therapy. The work presented in this thesis used a novel toxic retroviral vector to conduct a genetic screen to identify mutated V79-4 cell lines resistant to retroviral-mediated transduction. The toxic gene, barnase, was derived from *Bacillus amyloliquefaciens* and the toxicity could be suppressed stoichiometrically via a potent inhibitor, barstar, also derived from the same bacterium (Hartley, 1988). Once the resistant cell lines were identified (31-2 and 67-1), they were characterized and the level of resistance was quantified. Generally, 31-2 was approximately 5-10 fold resistant when compared to the parental wildtype V79-4 cells while 67-1 was approximately 10-20 fold resistant. Using a technique developed and refined by the Genomics Institute of the Novartis Research Foundation (GNF) called GFAcT (Chanda et al., 2003), we identified a novel gene MRI (Modulator of Retroviral Infection) that made 67-1 once again susceptible to retrovirally mediated transduction.

When we began this study, we were attempting to isolate clonal populations of cells completely resistant to retroviruses. Since retroviral vectors behave similarly to retroviruses, we utilized an MLV-based retroviral vector to recapitulate the early retrovirus life cycle. We chose an MLV-based retroviral vector to serve as our conduit for the toxic vector because it is a simpler retrovirus and we wanted to understand the most basic and foundational mechanisms of retrovirally mediated entry and expression. Additionally, by using a simpler retrovirus as our delivery mechanism we expected to

identify distinct populations of resistant cell lines. Ideally, these populations would have fallen into three distinct categories: 1) those completely resistant to MLV, but susceptible to lentivirus, 2) those completely resistant to both retroviral vectors and lentiviral vectors, and 3) cell populations only partially resistant to retrovirally mediated transduction. Depending on the category of resistance identified, different techniques would be used to identify the gene conferring the resistance phenotype. Although we only found clonal populations of cells partially resistant to retrovirally mediated transduction, I will discuss strategies for identifying genes responsible for all three types of mutations.

Cell Line Resistant to MLV, but susceptible to Lentivirus

If a cell line was identified that was completely resistant to MLV-based vectors, but susceptible to lentiviral vectors it would have been relatively easy to determine the gene conferring that resistance. Complementation cloning with a lentiviral cDNA library could be used to identify the gene. We would generate a cDNA library from the wildtype cell, place it within a lentiviral vector, and infect the MLV resistant cell line. Then we could infect the resistant MLV cell line with an MLV-based vector encoding a selectable marker (like *puromycin* or *neomycin*), add the drug, and then identify any clonal populations of cells that emerge. The cDNA that now made the previously resistant cell line susceptible could be identified via PCR. This technique of complementation cloning with a virally based cDNA library has previously been used to identify co-receptors necessary for SIV (Deng et al., 1997) infection and identify of members of the TNF (Sasazuki et al., 2002) or Fas (Perez et al., 2002; Somia et al., 1999) mediated pathway. Of all the potential mutants that could be identified in the screen, a

mutant resistant to MLV but susceptible to lentivirus (or vice versa) would lend itself to the easiest mechanism of gene identification, complementation cloning with a lentiviral cDNA library.

Cell Line Resistant to Retroviral and Lentiviral vectors

If the cell line was completely resistant to retrovirally mediated transduction the ability to use retroviral-based cDNA libraries to conduct complementation cloning is lost. However, another method exists to conduct complementation cloning non-virally. If we had discovered a cell line completely resistant to both retrovirally and lentivirally mediated transduction we could have created a cDNA library in the Tc-1 like transposon vector system, Sleeping Beauty (Ivics et al., 1997). The transposon is flanked by IR/DR repeat elements and the transposase utilizes these elements to initiate a "cut and paste" mechanism whereby the transposon is inserted randomly into a TA dinucleotide into the host chromosome (there is small but significant bias toward genes and their regulatory sequences) (Yant et al., 2005). The transposon/transposase system is active in a wide range of cell types and is capable of greater than 10,000 transposition events with a single transposon/transposase transfection (Izsvak et al., 2000). More recently, groups have made changes to the transposon base to increase payload size (Zayed et al., 2004) and created a hyperactive transposase (SB100X) that significantly increases transposition frequency (Mates et al., 2009). Additionally, optimizing the amount of DNA to transfect along with the proper transposon to transposase DNA ratio also increases transposition frequency. Although the gene transfer frequency is relatively lower compared to viral vectors, multiple transfections into multiple plates would allow saturation of the mutated

cell line with SB mediated transposition events. Ideally, one transposition event would complement the resistance phenotype and allow the cell to be susceptible to retrovirally mediated transduction. The transposed populations would then be infected with a retroviral vector carrying a selectable marker and the pro-drug applied to the cells. The now susceptible clonal populations would grow out and the transposed gene restoring infectivity could then be identified through PCR. The non-viral Sleeping Beauty transposon is an ideal system to identify genes that confer resistance to retrovirally mediated transduction, since complementation cloning with retroviral vectors is not possible.

Cell Line Partially Resistant to Retroviral and Lentiviral Vectors

If a cell line identified was only partially resistant, it would be more difficult to identify the gene conferring this partially resistance phenotype. In the various mutagenesis studies that have been conducted, not a single cell line has been identified that has been completely resistant to retrovirally mediated transduction (Bruce et al., 2005; Gao and Goff, 1999). Our studies also only generated refractory clones. The clones that we identified, 31-2 and 67-1 were minimally five fold and up to forty fold resistant to retrovirally mediated transduction. This level of resistance prevents the use of either viral or transposon-based complementation cloning since the residual level of infectivity would lead to an unmanageable level of clones to analyze. Therefore, alternatives to complementation cloning would be needed to identify genes conferring partial resistance.

One technique that can be used to identify the gene(s) that confer partial resistance is SIB selection (McCormick, 1987). In this strategy, a cDNA expression library is fractionated and these fractions are transfected into the partially resistant cell line. These pooled transfected populations are then subjected to quantitative analysis (FACS and/or Luciferase) to determine if any are more susceptible to infection than the parental resistant line. The cDNA fraction(s) which results in greater susceptibility are sub-fractionated, thereby further enriching for the gene(s) conferring susceptibility to the resistant cell line and quantitative analysis is once again performed on these pooled transfected populations. Further fractions are made of the sub-fractions to continually enrich for the gene(s) of interest, until the gene is identified. This technique has been used to identify a murine fibrogenic lymphokine in cell culture (Prakash et al., 1995) and more recently has been used in functional genomic studies in *Xenopus* to identify genes in neurogenesis (Voigt et al., 2005) and kidney development (Kyuno et al., 2008). Ultimately, we did not choose SIB selection because the phenotype of partial resistance was too high and the ability to utilize SIB selection would have been limited.

Another technique that could be used to identify genes that confer partial resistance to retrovirally mediated transduction is gene expression profiling with microarrays. This technique was first used to measure differential expression of 45 *Arabidopsis* genes (Schena et al., 1995). Now this technique is capable of quantifying gene expression levels across tens of thousands of genes and entire genomes (Lashkari et al., 1997). Microarray technology is commonly being used to quantify gene expression differences between diseased and non-diseased tissues in cancer biology (Golub et al., 1999; Pusztai, 2008). The hope is to identify genes responsible for the development of

cancer. This type of profiling is no different from mutated cells resistant to retrovirally mediated transduction ("affected") and the parental cell line susceptible to retrovirally mediated transduction ("unaffected"). Utilizing mouse or human microarrays available from Affymetrix (each microarray containing nearly all known human or mouse genes) and RNA harvested from both the partially resistant cell line and the parental cell line, gene expression profiles can be derived for each cell line and a comparison between the two expression profiles could identify the gene(s) rescuing the resistance phenotype. We chose not to utilize microarray technology for several reasons. First, at the time of our study, a hamster microarray did not exist. Second, we were concerned that comparing the heavily mutagenized 67-1 cells to wildtype V79-4 cells would result in far too many differentially expressing genes to analyze. In a later study, a group was able to identify a Rat gene conferring retroviral resistance when they compared gene expression profiles between mutated and wildtype cells (Naghavi et al., 2005). However, this group was able to identify their gene because they used a Rat specific mircroarray and their gene happened to be in subset of genes found on that Rat microarray.

A final technique that can be utilized to identify a gene rescuing partial resistance to retrovirally mediated transduction is GFAcT (Genome Functionalization through arrayed cDNA Transduction). GFAcT has been utilized previously to identify novel interactors of AP-1 (Chanda et al., 2003) and CREB (Conkright et al., 2003) by cotransfection of a cDNA expression library along with a construct expressing luciferase under control of either the AP-1 or CREB promoter. GFAcT has also been used to identify novel activators of Kaposi Sarcoma-Associated Herpesvirus by transfecting a cDNA expression library into cells harboring a latent form of the virus (Yu et al., 2007).

Using this method the Genomics Institute of the Novartis Foundation (GNF) analyzed over 11,000 unique human and mouse cDNAs for complementation in a transient overexpression system to identify genes that made our partially resistant cell line have greater susceptibility to retrovirally mediated transduction.

For multiple reasons, we ultimately chose GFAcT as our strategy to identify genes conferring resistant in 67-1 cells. SIB selection was unfeasible given the background level of infectivity. Since 67-1 was 20 to 40 fold resistant to retrovirally mediated transduction, this would mean for every 10 cm plate containing 1 x 10^6 cells transfected with a fractionated cDNA library, approximately 25,000 to 50,000 cells would survive even if they did not possess a transfected cDNA. The signal to noise ratio was therefore unacceptable. GFAcT was ultimately chosen because our collaborators at GNF were experts in its application since they had both developed and refined the technology. Additionally by using GFAcT, two distinct populations of genes could be identified. First, we could identify the mutated gene in the resistant line by complementation with a wild-type copy. Second, we could identify genes that act as overexpression suppressors of the mutation in our resistant cell line, while not actually complementing the mutated gene. Given these possibilities we chose GFAcT as our strategy to identify genes that could restore infectivity to 67-1.

Is it possible to generate a clonal population of cells completely resistant to retroviral infection?

Of the various cell lines that have been isolated and shown to be resistant to retrovirally mediated transduction, including the ones presented in our studies, not a

single clone has been shown to be completely resistant. Bruce and colleagues derived CHO cells that were 50 fold resistant to MLV, but completely susceptible to HIV (Bruce et al., 2005). Gao and Goff isolated populations of Rat-2 cells that were 100 to 1000 fold resistant to all retroviral-mediated transduction (Gao and Goff, 1999). Additionally, of all the genes conferring resistance to retrovirally mediated transdction that have been identifed through genetic screens, not a single one has been shown to create a completely resistance phenotype. One study showed that 30 fold overexpression of FEZ1 (fasciculation and elongation protein ζ-1), a protein kinase C ζ-interacting protein homologous to the *Caenorhabditis elegans* synaptic transport protein UNC-76, led to a 1000 fold resistant cell line (Naghavi et al., 2005). Gao and colleagues, building on their previous study (Gao and Goff, 1999), showed that expression of rZap (rat Zinc-finger Antiviral Protein) caused Rat-2 cells to become 30 fold resistant to retrovirally mediated transduction (Gao et al., 2002). Most recently, Wolf and Goff showed that embryonic stem cells utilize zinc finger protein ZFP809 to transcriptionally silence MLV and HTLV-1 and that expression of ZFP809 in differentiated cells induces a 14 fold resistance to MLV and HTLV (Wolf and Goff, 2009).

Two families of host cell factors facilitate near complete resistance to retrovirally mediated transduction. A family of restriction factors deemed *Fv1*, *Ref1*, and *Lv1* all inhibit retroviral replication at a step following membrane fusion and preceeding integration. *Fv1*, a mouse gene with homology to endogenous retroviral *Gag*, causes resistance to N or B tropic strains of MLV(Best et al., 1996). *Ref1* (later determined to be the human homolog of Trim5α), a gene found in many mammalian species, including humans, restricts N-tropic MLV (Towers et al., 2000). *Lv1* (ultimately determined to be

a Trim5α homolog), found in non-human primates, inhibits HIV-1 replication (Stremlau et al., 2004). These three restriction factors show extremely high levels of resistance, but this resistance is easily overcome by using large quantities of restricted virus or virus-like particles (Bieniasz, 2003). Another restriction factor, APOBEC3G, was characterized as a DNA deaminase that incorporates into virions during viral production and induces deamination of deoxycytidine to deoxyuridine (Harris et al., 2003). This deamination results in hypermutations from G to A during the process of reverse transcription of the viral mRNA, leading to degradation of the viral genome and near complete inhibition of HIV-1 infection (Harris et al., 2003; Mangeat et al., 2003). However, this host defense is overcome by the HIV accessory protein Vif which targets APOBEC3G for degradation via the cellular proteasome (Sheehy et al., 2002).

There are several possibilities that explain why not a single clonal population of cells has been shown to be completely resistant to retrovirally mediated transduction. First, it may not be possible to isolate a population of cells completely resistant to viral infection. Since retroviruses behave as obligate intracellular parasites and harness the cellular machinery to complete its lifecycle, the retrovirus may only interact with machinery that is critical for cellular survival. Eliminate a component needed by the retrovirus to complete its lifecycle and you also eliminate a component necessary for cellular survival. Given the relatively simplistic organization of retroviruses, as even the most complex genus (lentivirus) is only composed of fifteen proteins (Frankel and Young, 1998), it is reasonable to assume that the basic machinery for retrovirus replication is essential for cellular survival.

Another possible explanation for the lack of a completely resistant clone is genetic redundancy. Through genome wide *Saccharomyces cerevisiae* studies, it has been shown that minimally one quarter of the yeast genes can be singly deleted with no phenotypic effect (Gu et al., 2003). This is predominately attributed to the existence of duplicate genes (i.e. the loss of function in one copy can be compensated by the other copy or copies) or alternative metabolic pathways/regulatory networks. Given that a retrovirus utilizes basic cellular functions to complete its lifecycle, it is reasonable to speculate that these basic cellular proteins and/or pathways have some level of redundancy since they are also essential for cellular survival. Therefore, if you eliminate the primary protein/pathway utilized by HIV and the cell, a less utilized protein or secondary pathway can compensate, albeit in a much less efficient manner, resulting in a partially resistance phenotype. If this secondary protein or pathway is utilized less efficiently this could also lead to reduced fitness. This is consistent with what we observed with both the 31-2 and 67-1 mutant cell lines. Both these cell lines were only partially resistant to retroviral-mediated transduction and both had a slightly reduced fitness as determined by growth rate. In both 31-2 and 67-1, compensation by a secondary protein or pathway could be causing a partially resistance phenotype and preventing the isolation of a completely resistant cell line.

Two separate experiments can be used to determine if genetic redundancy is causing the lack of completely resistant clones. First, cell fusions between different resistant lines, with each resistant line possessing a fluorescent marker with a unique fluor, are created and these fused cells are challenged with a retroviral vector encoding a third fluorescent tag. Flow cytometry analysis is conducted to analyze for the frequency

of all three colors, i.e. fused cells which are also infected. Using the cis-trans test for genetic complementation, if the mutations in the fused resistant cell lines are in different pathways or proteins, also assuming the mutations are recessive, then we would expect a high level of infectivity by the retroviral vector. However, if after fusion the infectivity rate is similar to the resistant cell lines, then the mutations in those cell lines do not complement each other and may be in the same pathway or affect the same protein (or one of the mutant cells lines has a dominant mutation). After identifying the genes conferring partial resistance with either SIB selection, Affymetrix chip analysis, or GFAcT, sequence analysis can determine if the mutations are in the same gene or potentially in the same pathway. Another mechanism to examine for genetic redundancy is to perform mutagenesis on a partially resistant cell line. If the primary pathway or protein was eliminated in the first round of mutagenesis, the secondary pathway or protein may be eliminated in this subsequent mutagenesis (assuming that this pathway or protein is not essential for cellular survival). With these two methods, it is possible to first determine if genetic redundancy is preventing the creation of a cell line completely resistant to retrovirally mediated transduction and then potentially create a completely resistant cell line with additional mutagenesis.

Another possible explanation for the lack of a clonal population of cells completely resistant to retrovirally mediated transduction is non-saturation of the initial mutagenesis screen. To determine if a screen is saturated, first you must determine the frequency of targeting the same dizygous gene in the screen (Pollock and Larkin, 2004). We looked for the frequency of APRT deficient clonal populations of cells. We observed a number of cells (23 in 10^7 4^{th} round mutagenized cells vs. 0 in wildtype cells) which

were unaffected by diaminopurine (DAP, a drug which is converted to a toxic nucleotide by APRT). This indicated that our screen was saturated since we were able to elicit DAP resistance at a relatively high frequency. However, we did not determine if the APRT gene was actually targeted by our mutagen or if DAP resistance was the result of a failure to take up the drug or some other process indirectly related to DAP processing. Any of these mechanisms may have artificially inflated our recovery rate of resistant clones. To further determine saturation of the screen, sequencing of the APRT gene in multiple resistant clones would be necessary. The greater the number of different mutations found in the APRT gene, the greater the saturation point of the screen. If the same mutation was found in all 23 DAP resistant clones, this would indicate a relatively low level of saturation, but higher levels of saturation would be indicated with increasing numbers of unique mutations (Pollock and Larkin, 2004). If our screen was not sufficiently saturated, that too could be a reason why we did not identify a clonal population of cells completely resistant to retrovirally mediated transduction.

What is the mutation in 67-1?

Although we did not identify the mutation in 67-1 that caused the resistance phenotype, we did identify MRI in our GFAcT screen. MRI, when transfected into 67-1, behaves as an overexpression suppressor and causes 67-1 to once again be susceptible to retroviral infection at rate comparable to wildtype V79-4 cells. We ultimately showed that knockdown of endogenous MRI can recapitulate a resistance phenotype and that MRI may modulate retroviral infection by playing a role in proteasome degradation to facilitate viral uncoating or proteasome degradation of the virus itself. The identification

of MRI in our screen was validated when our collaborators at GNF performed a large scale functional genomics screen to identify host cell factors which interact with HIV (Konig et al., 2008). What makes this validation significant is that Konig and colleagues utilized a different methodology. While our screen looked for genes that restored infectivity by expressing genes in a resistant cell line, the Konig screen identified genes that conferred resistance when knocked down with siRNAs. Confirming our results, MRI was identified in their screen and knockdown of MRI in their hands resulted in knockdown of infectivity of 40 to 42% relative to controls. The relative viral inhibition seen by Konig and colleagues compares very favorably to our shRNA knockdown experiments that resulted in infectivity levels of 26% and 39% as compared to wildtype (Fig. 3-11B).

Although we did not identify the mutated gene(s) in 67-1 that conferred the partially resistance phenotype, identification of the gene(s) can be accomplished with two techniques. First, we can repeat the GNF GFAcT screen. In order for this methodology to work a human and/or mouse gene must be able to complement the hamster mutant. This is likely given that human MRI acts as an overexpression suppressor in a hamster cell line. When we first performed our screen, we only tested 67-1 against 11,000 unique human and mouse cDNAs. Estimates have put the number of mouse genes at around 21,500 (Zhang et al., 2004) and human genes at around 20,500 (Clamp et al., 2007). Therefore, we only screened 67-1 against about one quarter to one-half of the combined predicted human and mouse genes. It is possible that our gene was not present in our initial screen. If we repeated the screen today, we could potentially screen against every known mouse and human gene since GNF now possesses plasmid cDNA collections of

19,832 unique human cDNAs from Origene, 20,739 unique human cDNAs from the Mammalian Gene Consortium, and 15,652 unique mouse cDNAs from the Mammalian Gene Consortium. Given these larger numbers of genes, we increase the likelihood of finding the mutated gene in 67-1.

Another method that can be utilized to identify the mutation in 67-1 would be to conduct gene chip analysis and identify the genes in 67-1 based on differential expression between the wildtype V79-4 and mutant 6701 cells. In order for this methodology to work, hamster genes must hybridize with mouse genes, as Affymetrix does not make a commercially available hamster gene chip. This concern has been alleviated since a recent study has indicated that existing Affymetrix mouse oligo-arrays can be used successfully for gene profiling of hamster cells (Yee et al., 2008). Furthermore, if we are still concerned with hamster/mouse hybridization, Affymetrix can generate custom CHO microarrays utilizing hamster EST sequences described in a previous study (Wlaschin et al., 2005). By simply harvesting total RNA from both mutated 67-1 cells and wild-type V79-4 cells and generating cDNA from these pools, gene expression profiling can be conducted with the appropriate Affymetrix gene chip. Differentially expressed genes can be identified and subjected to further analysis to determine if they are mutated in 67-1.

Finding HIV therapeutic targets

Numerous host cell proteins have been identified that play a role in HIV infections. These host cell proteins are potential targets for novel therapies since the rapid mutation rate of HIV and high viral load facilitate the appearance of virions which are resistant to the drugs that specifically target HIV proteins. However, not all of these

host cell proteins are ideal since they may not be druggable or they are essential for cell survival. Therefore, the identification of non-essential, druggable host cell proteins is critical. Loss of function genetic screens, as the one conducted to identify MRI, represent one strategy to identify non-essential host cell proteins necessary for productive HIV infections. The use of genetic screens to identify these non-essential host proteins is the first step toward the creation of novel HIV therapies.

CHAPTER 5

Materials and Methods

Table 5-1: Abbreviations

Ampho- Amphotropic 10A1
APRT- Adenine Phosphoribosyltransferase
β-gal- Beta Galactosidase
Bsr- Blasticidin Resistance
BST-2- Bone Marrow Stromal Cell Antigen 2
CA- Capsid
Cdk9- Cyclin Dependent Kinase 9
cPPT- central Polypurine Tract
CMV- Cytomegalovirus
C_t- Cycle Threshold
CycT1- Cyclin T1
DHFR- Dihydrofolate Reducatase
DMEM- Dulbecco's Modified Eagle's Medium
Dox- Doxycycline
dsRed- Red Fluorescent Protein derived from *Discosoma sp.*
EF1α- Elongation Factor 1α
EGFP- Enhanced Green Flourescent Protein
FACS- Flow Assisted Cell Sorting
FasL- Fas Ligand
FDA- Food and Drug Administration
FEZ1- Fasciculation and Elongation Protein ζ-1
FIV- Feline Immunodeficiency Virus
GFAcT- Genome Functionalization through cDNA Transduction
HAART- Highly Active Antiretroviral Therapy
HIA- Heat Inactivated
HIV- Human Immunodeficiency Virus
HMGA1- High Mobility Group Protein A1
HPRT- Hypoxanthine Phosphoribosyltransferase
HSV-*TK*- Herpes Simplex 1 Thymidine Kinase
HTLV- Human T-Cell Leukemia Virus
Int- Integrase
IU- Infectious Unit
LEDGF- Lens Epithelium Derived Growth Factor
LTR- Long Terminal Repeat
MA- Matrix
MAPK- Mitogen Activated Protein Kinase
MHC I- Major Histocompatibility Class I
miRNA- micro RNA
ml- milliliters

Table 5-1: Abbreviations (continued)

MLV- Moloney Leukemia Virus
m.o.i.- Multiplicity of Infection
MRI- Modulator of Retroviral Infection
MVB- Multivesicular Body
NES- Nuclear Exportation Signal
NC- Nucleocapsid
NLS- Nuclear Localization Signal
nNRTI- non-Nucleoside Reverse Transcriptase Inhibitors
NRTI- Nucleoside reverse Transcription Inhibitors
PAGE- Polyacrylamide Gel Electrophoresis
PBS- Phosphate Buffered Saline
PBST- Phosphate Buffered Saline Tween-20
PCR- Polymerase Chain Reaction
PI- Protease Inhibitor
PI3K- Phosphoinositide 3-Kinase
PIC- Pre-integration Complex
Pro- Protease
PSA- Prostate Specific Antigen
RCL- Replication Competent Lentivirus
RCR- Replication Competent Retrovirus
RLU- Relative Light Units
RNAi- RNA interference
RNAP II- RNA Polymerase II
RRE- Rev Response Element
RT- Reverse Transcriptase
shRNA- short hairpin RNA
SIN- Self-Inactivating
siRNA- small interfering RNA
SIV- Simian Immunodeficiency Virus
TAR- Transactivation Response
TFT- Trifluorothymidine
TNF- Tumor Necrosis Factor
TSG101- Tumor Suppressor Gene 101
VSV-G- Vesicular Stomatitis Virus G-protein
wt- wildtype

Cell Lines Used

All cultures were maintained in DMEM supplemented with 10% FBS and 50 units/ml each of penicillin and streptomycin unless otherwise indicated.

Table 5-2: Cell Lines Used

Name	ATCC #	Origins
A-498	HTB-44	Male human kidney carcinoma
DU-145	HTB-81	human prostate cancer
Hela	CCL-2	human cervical cancer epithelial
HOS	CRL-1543	female human osteosarcoma
MRC-5	CCL-171	Male human fetal lung fibroblast
U87	HTB-14	female human glioma cell
V79-4	CCL-93	Male hamster lung fibroblast
31-2	n/a	derived from V79-4
67-1	n/a	derived from V79-4
293T	CRL-11268	female embryonic kidney

Table 5-3: Primers Used

Primer Name	Purpose	Sequence (5' to 3')
CSII Barnase (F)	Cloning Barnase into CSII	GGCCTCGAGCCATGGCACAGGTTATCAACAC
CSII Barnase (R)	Cloning Barnase into CSII	CTCGGATCCTTATCTGATTTTTGTAAA
MLV Barnase (F)	Cloning Barnase into CLMFGv2	GAGCGCTAGCCATGGCACAGGTTATCAACAC
MLV Barnase (R)	Cloning Barnase into CLMFGv2	GGACTCGAGTTATCTGATTTTTGTAAA
pRK-Barstar (F)	Cloning Barstar into pRK5	CCCGAATTCCATGGGCAAAAAAGCAGTCATTAA
pRK-Barstar (R)	Cloning Barstar into pRK5	CCCAAGCTTTTAAGAAAGTATGATGGTGAT
β-cctin (F)	quantitative PCR control	ATATGTTTGAGACC TTCAA
β-actin (R)	quantitative PCR control	AGATGGGCACAGTGTGGGT
U31	quantitative PCR for HIV RT product	GGATCTACCACACACAAGGC
U32	quantitative PCR for HIV RT product	GGGTGTAACAAGCTGGTGTTC
LTR9	quantitative PCR for HIV RT product	GCCTCAATAAAGCTTGCCTTG
5NC2	quantitative PCR for HIV RT product	CCGAGTCCTGCGTCGAGAGAGC
AA55	quantitative PCR for HIV RT product	CTGCTAGAGATTTTCCACACTGAC
qMRI-1 (F)	quantitative PCR for MRI-1	GCTCTGGGAATCCTGATTGA
qMRI-1 (R)	quantitative PCR for MRI-1	TGGGAAGGGCTGAGGC
mMRI-1 (F)	Amplification of Hamster MRI-1 cDNA	AGCGAATTCATGGAAACCCTAAAATCC
mMRI-1 (R)	Amplification of Hamster MRI-1 cDNA	GCCCGATCCCTAACTGAAAAATATTTCCCT

Table 5-4: Plasmids

Name	Purpose	Origin
CLMFG-Barnase	MLV vector transducing Barnase	Bryan Nikolai
pCLMFG-EGFP	MLV vector transducing EGFP	Nikunj Somia
pCLMFGv2	MLV cloning vector based on pCL vectors	Nikunj Somia
CMVgp	*Gag*, *Pol*, and *Rev* of MLV expression	Nikunj Somia
CSII-Barnase	HIV-1 vector transducing Barnase	Bryan Nikolai
CS-CDF-CG-PRE	HIV-1 vector transducing EGFP (CMV promter)	Tomoyuki Yamaguchi
CS-CDF-EG-PRE	HIV-1 vector transducing EGFP (EF1α promter)	Tomoyuki Yamaguchi
CSII-3xFlag-MRI	HIV-1 vector tranducing MRI with 3x N-terminally fused Flag	Sumit Agarwal
CSII-Bsr-EGFP	HIV-1 vector transducing Blasticidin/EGFP fusion protein	Tomoyuki Yamaguchi
CSII-EF-dsRed	HIV-1 vector transducing dsRed	Sumit Agarwal
CSII-EF-EGFP	HIV-1 vector transducing EGFP (EF1α promter)	Tomoyuki Yamaguchi
CSII-EF-MCS	EF1α promoter driven HIV-1 cloning vector	Tomoyuki Yamaguchi
CSII-Luciferase	HIV-1 vector transducing Luciferase	Sumit Agarwal
pGL3	Luciferase expression	Promega (Madison, WI)
pMDG	VSVG envelope expression	Nadini, et. al. 1996. Science 272:263-7.
pMT416	Barnase and Barstar expression	Hartley, et. al. 1972. Prep. Biochem 2:229-250.
N-GFP-MRI	Expression construct for N-terminal GFP fused MRI	Sumit Agarwal
ΔNRF	*Gag*, *Pol*, and *Rev* of HIV-1 expression	Gift from Tal Kafri (University of NC)
pRK5	Cloning expression vector	BD Biosciences (San Jose, CA)
pRK-Barstar	Barstar expression	Sumit Agarwal
pRK5-EGFP	EGFP expression	Nikunj Somia
pRK5-10A1	Amphotrophic envelope expression	Nikunj Somia
pPUR	Puromycin expression	Clontech (Mountain View, CA)

To generate CSII-barnase, the coding sequence of barnase was amplified from pM4TI6 (Hartley and Rogerson, 1972; Hartley et al., 1972) using primers CSII-Barnase (F) and CSII-Barnase (R) and cloned into CSII-EF-MCS at the *Xho*I and *Bam*HI sites.

To generate CLMFG-barnase, the coding sequence of barnase was amplified from pMT416 (Hartley and Rogerson, 1972; Hartley et al., 1972) using primers MLV-Barnase (F) and MLV-Barnase (R) and cloned into pCLMFGv2 using *Nco*I and *Xho*I cloning sites.

To generate pRK5-barstar, the coding sequence of Barstar was amplified from pMT416 (Hartley and Rogerson, 1972; Hartley et al., 1972) using the primers pRK5-Barstar (F) and pRK5-Barstar (R) and cloned into the pRK5 using *EcoR*I and *Hind*III cloning sites.

To generate CSII-luciferase, the coding sequence of luciferase was isolated from the pGL3 vector (Promega; Madison, WI) via an *Nco*I (blunted) and *Xho*I digestion. This fragment was then cloned into CSII-MCS-EF using *Eco*RI (blunted) and *Xho*I cloning sites.

Mutagenesis

Ten plates of V79-4 at a density of $1x10^6$ cells per 10 cm plate were mutagenized for 10 hours using 10 μg/ml ICR-191 (Sigma; St. Louis, MO). The medium was changed and the cells were allowed to recover to confluency. Mutagenesis was repeated as described above for a total of four rounds. Efficacy of mutagenesis was determined by observing the number of $HPRT^-$ and $APRT^-$ clones from 10^7 cells. $HPRT^-$ clones were observed by selection with 6-thioguanine (Sigma; St. Louis, MO) at 10 μg/ml, and

APRT$^-$ clones were observed by selection with Diaminopurine (Sigma; St. Louis, MO) at 50 μg/ml.

Retroviral Vector Production

MLV and HIV vectors were generated as previously described (Naldini et al., 1996) by transfecting various amounts of DNA using the methods of Chen and Okayama (Chen and Okayama, 1987). Two different envelope plasmids were utilized; pMDG encoding the VSV-G protein and pRK5-10A1 encoding the MLV 10A1 amphotrophic envelope.

To generate MLV vector, 15 μg vector DNA, 10 μg CMVgp, 5 μg pMDG, and 2 μg pRK5-EGFP were transfected into a 10 cm dish that was plated at a density of 5 x 10^6 293T cells 24 hours earlier. If MLV based barnase was being generated, 15 μg of pRK5-Barstar was also transfected. Approximately 12 hours post transfection, the medium was changed. Seventy-two hours after transfection virus was collected, filtered through a 0.45 μm filter, and stored in aliquots at -80^o C.

To generate lentiviral vector, 15 μg vector DNA, 10 μg ΔNRF, 5 μg pMDG or 5 μg pRK5-10A1, and 2 μg pRK5-EGFP were transfected into a 10 cm dish that was plated at a density of 5 x 10^6 293T cells 24 hours earlier. If lentiviral based barnase was being generated, 15 μg of pRK5-Barstar was also transfected. Approximately 12 hours post transfection, the medium was changed. Seventy-two hours after transfection, virus was collected, filtered through a 0.45 μm membrane, and stored in aliquots at -80^o C.

Infective titers for EGFP-transducing vector were determined by infecting 10^5 HeLa cells with dilutions of the virus preparation. The medium was changed after 12 hours of incubation of the virus preparation with the cells. The extent of EGFP

expression was quantified 72 hours after infection by flow cytometry on a Becton-Dickinson FACScan (San Jose, CA) with subsequent analysis using Becton-Dickinson CellQuest 3.1 software (San Jose, CA).

Lentiviral vectors that were utilized for qPCR were treated with 25 U/ml DNase I at room temperature for 1 hour to eliminate all plasmid DNA. As a qPCR control for DNA contamination, DNase I-treated virus was placed in a boiling water bath for 30 minutes to serve as the heat inactivated sample.

Crystal Violet Incorporation Assay for Determining Growth Rate and Barnase Vector Mediated Cell Death

For growth rate determination, 10^4 cells/well of the relevant cell type were plated into 24 well plates. At given time-points cells were washed twice with 1X PBS, stained with 0.1% crystal violet in 70% methanol, rinsed with water to remove unincorporated crystal violet, and allowed to dry. Each time point was performed in quadruplicate.

For quantification of barnase mediated cell death 10^4 cells/well of the relevant cell type were plated into 24 well plates. After overnight incubation, the wells were infected with two fold serial dilutions of virus in a total volume 1 ml (viral supernatant being generated plus and minus pRK5-barstar). Each dilution point was performed in quadruplicate. The next day the virus was removed and replaced with 1 ml of media. Forty-eight hrs later, the plates were washed twice with 1X PBS, stained with 0.1% crystal violet in 70% methanol, rinsed with water to remove unincorporated stain, and allowed to dry.

For both the growth curve and cell death analysis, the incorporated crystal violet was eluted with 1 ml of 100% methanol, transferred to a cuvette, and the absorbance was

determined at 490 nm by a Beckman DU 640B spectrophotometer (Fullerton, CA). The quadruplicated absorbance values (which measured incorporated crystal violet) were averaged and plotted in Excel versus 1) time (for growth curve) or 2) amount of virus (for cell death).

Colony Count Assay

10^5 cells/well were plated onto six well dishes and, after overnight incubation, infected with 1 μl or 10 μl of CSII-Bsr-EGFP lentiviral vector in a total volume of 2 ml. Twenty-four hours later each well was subcultured into 10 cm dishes at $1x10^4$ cells per plate with selective medium containing 250 μg/ml Blasticidin (Invivogen**;** San Diego, California). After 10 days (or when individual colonies were visible), the colonies were stained with 0.1% crystal violet in 70% methanol and counted. As a parallel experiment, 20 μg of the CSII-Bsr-EGFP fusion was transfected using the method of Chen and Okayama (Chen and Okayama, 1987). Blasticidin selection was applied 24 hours later, and the amount of gene transfer was determined after 10 days (or when individual colonies were visible) by staining and counting Bsr resistant colonies. Colony numbers were counted, plotted in Excel, and compared.

Luciferase Assay

10^5 cells/well were plated onto six well dishes and, after overnight incubation, the cells were infected with varying amounts of CSII-luciferase lentiviral vector in a total volume of 2 ml. Each dilution point was assayed in triplicate. Twelve hours after infection the medium was changed, and 72 hours after infection the cells were washed with once with 1X PBS and lysed using Reporter Lysis Buffer following the manufacturer's protocol (Promega; Madison, WI). Luciferase activity was measured

using the luciferase assay system (Promega; Madison, Wisconsin) following the manufacturer's protocol on a Model Lumat LB9507 luminometer (Berthold Technologies**;** Oak Ridge, TN). Luciferase activity levels were averaged, and plotted in Excel comparing luciferase activity by cell type.

FACS Analysis

Cells were typically plated onto six well dishes at a density of 10^5 cells/well and allowed to recover overnight. The plates were then infected with a specific m.o.i. of virus (see Retroviral Vector Production for titer determination). The medium was changed 12 hours after incubation of the virus with the cells. The extent of reporter expression (EGFP or dsRed) was quantified 72 hours after infection by flow cytometry on a Becton-Dickinson FACScan (San Jose, CA) with subsequent analysis using Becton-Dickinson CellQuest 3.1 software (San Jose, CA).

Cell-Fusion Assay[8]

Cells were plated 5 x 10^6 cells on 10-cm dishes, and after 18 hours they were stained for 20 minutes with Oregon green (O34550; Invitrogen, Carlsbad, CA) or Vybrant DID (V22887; Invitrogen) probes according to the manufacturer's protocol. The cells were washed three times with 1X PBS and allowed to recover for four hours. The cells were removed from the plate with a non-trypsin dissociation medium, and self-self or V79-4 and 67-1 cells were mixed in 15 ml conical tubes (Falcon, Brookings, SD) and concentrated by centrifugation for 5 min at 500 x g. To the pellet was added 1 ml of sterile 50% PEG 3000-3700 (Sigma, St. Louis, MO) and 2% glucose in PBS solution; after 1 min, 1 ml of 1X PBS was added to the cells. After 45 seconds, 3 ml of 1X PBS + 2% FBS was added to the cells, and cells were washed twice with pelleting (5 min at 500

[8] Another member of the lab, Patrycja Lech, conducted this experiment.

x g) and resuspended in 1X PBS + 2% FBS. Cells were plated (in DMEM without phenol red + 20% FBS) onto two 10-cm tissue culture dishes and allowed to recover for 6 hours. At this time the cells were infected with a CSII-EF-dsRED lentiviral vector at an m.o.i. of 1 (infection units calculated on HeLa cells). The cells were analyzed 48 hours later by flow cytometry using four-color differentiation on a FACSCalibur with subsequent analysis using CellQuest Pro acquisition software. Background leakage through the channels was compensated by subtraction of the background value from all samples.

Reverse transcription product qPCR assay

3.5 x10^5 cells were plated onto six well dishes and infected at an m.o.i. of 0.5 with DNase I treated lentiviral vector. Cells were incubated with virus for 6, 12, 24, or 36 hours. Controls consisted of mock infection or HIA vector samples incubated with cells for 36 hours. At the indicated time points, wells were washed twice with 1X PBS, harvested into an eppendorf tube and pelleted. Total cell lysates were prepared by resuspending the cell pellet in lysis buffer (Tris pH 8.0, 25 mM EDTA pH 8.0, 100 mM NaCl, 1% Triton X-100, and 2 mg/ml proteinase K) and incubating at 55 ^{o} C overnight. The next day, the proteinase K was heat inactivated by boiling the sample for 15 minutes. Lysates were used as templates for PCR analysis.

The following primers were used for qPCR (Zack et al., 1990): β-actin (F), β-actin (R), U31, U32, LTR9, 5NC2, and AA55. Primers LTR9 and AA55 were used to quantitate strong stop product, U31 and U32 for 1st jump product, and primers LTR9 and 5NC2 for full product. Quantitative PCR reactions using SYBR green were performed using a Biorad iCycler equipped with an optical module and BioRad SuperMix (without

ROX) following the manufacturer's protocol **(**Biorad; Hercules, CA). PCR reactions contained 1 μl template, 1.5 μl each primer pair (10 mM), 12.5 μl SuperMix and 8 μl dH_20. Cycling conditions used were 95 ^{o} C for 3 min, followed by 40 cycles of 95^o C 30sec, 58 ^{o} 30sec, and 72 ^{o} 30sec, and a final extension (5 min 72 ^{o} C) to complete all the PCR products.

qPCR analysis was conducted with the Biorad software. The melt curve generated by the Biorad software indicated the presence of a single PCR product (data not shown) and agarose gel electrophoresis confirmed the presence of a single product at the expected size (data not shown). Quantification of the lentiviral cDNA and β-actin was determined from the cycle threshold (C_T). β-actin expression was quantified using an aliquot of the same lysate that was used to generate the HIV cDNA data. The amount of HIV cDNA was than normalized to β-actin levels by generating a ΔC_T (C_T for the HIV cDNA – C_T for the β-actin gene fragment). Generation of ΔC_T values allows for comparison between different cell lines. A numerical value of five was added to all C_T values to generate a positive integer for data representation. Note that in these comparisons a lower C_T value reflects higher amount of lentiviral cDNA in the sample.

MG-132 Rescue Experiments[9]

MG-132 (BIOMOL, Plymouth Meeting, PA) was resuspended in DMSO and added at doses between 1 and 10 μM to 10^5 V79-4 or 67-1 cells for two hours. After this preincubation, cells were infected for 6 hours with an EGFP-marked lentiviral vector (CSII-EF-EGFP) at an m.o.i. of 0.5 (as measured on HeLa cells) in the presence of the inhibitor. Cells were washed twice in culture medium and incubated for 72 hours in

[9] Another member of the lab, Patrycja Lech, conducted this experiment.

medium without MG-132. After this time, the extent of infection was quantified by flow cytometry on a FACScalibur (BD Biosciences, San Jose, CA) with subsequent analysis using CellQuest Pro acquisition software (BD Biosciences).

Equilibrium Density Gradients[10]

This preparation was done essentially as described (Fassati and Goff, 2001). Cells (V79-4 and 67-1) were plated at 10^6 on two 10-cm dishes and allowed to recover overnight. The cells were then infected with CSII-EF-EGFP virus at an m.o.i. of 5 (as measured on HeLa cells), and the infection was allowed to proceed for 6 hours at 37°C. The cells were then collected in PBS, pelleted, and resuspended in five volumes of hypotonic buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 5 mM DTT, 20 μg of aprotinin per ml, 2 μg of leupeptin per ml). Cells were pelleted (16,000 x g) and resuspended in three volumes of hypotonic buffer. After 10 minutes on ice, cells were processed using a Dounce homogenizer, and the nuclear and membrane components were cleared by centrifugation for 15 minutes at 3,300 x g. The supernatant was further clarified by centrifugation for 20 minutes at 7,500 x g and loaded onto a 10 ml 20-70% sucrose gradient. Cell components were fractionated by centrifugation for 20 hours at 4°C at 30,000 rpm in a Sorvall SW40 swinging rotor (Thermo Electron Corporation, Asheville, NC). One-milliliter fractions were collected from the top, and 200 μl was saved for measurement of density to verify the integrity of the gradient. The protein in the remaining 800 μl was pelleted by the addition of trichloroacetic acid (10% final concentration), and the pellet was washed with ice-cold 100% acetone. The pellet was collected into PAGE loading buffer, and the proteins were separated by PAGE.

[10] Another member of the lab, Jeff Schreifels, conducted this experiment.

Immunoblot Analysis for p24[11]

Immunoblot analysis was performed as described (Somia et al., 1999). Mouse anti-p24 Ab (183-H12-5C) was obtained from Dr. Bruce Chesebro and Kathy Wehrly through the National Institutes of Health AIDS repository and used at a 1:3,000 dilution. Secondary Ab was HRP-conjugated goat anti-mouse IgG (Pierce, Rockford, IL), and detection was performed by using the Femto chemiluminescence reagents (Pierce) according to the manufacturer's instructions.

MG-132 Rescue of Capsid (p24) Stability[12]

V79-4 or 67-1 cells (5×10^5) were preincubated with 10 μM MG-132 and then infected with a CSII-EF-EGFP lentivirus at an m.o.i. of 0.5. At 1, 2, 4, and 6 hours, the cells were lysed, and immunoblot analysis was performed as described above.

High Throughput (Retro)Transfection and Reporter Assay[13]

High-throughput (retro)transfections of approximately 11,000 unique human and mouse full length cDNA expression constructs were spot arrayed in 384 well plates. The methodology using this type of a cDNA library has been previously described and used to identify novel effectors of AP-1 mediated growth (Chanda et al., 2003)**.** The (retro)transfection protocol is as follows: 20 μl of serum-free medium containing FuGENE 6 (Roche; Indianapolis, IN) was added to each well of a 384-well plate containing 62.5 ng of a distinct cDNA expression construct. 2×10^3 67-1 or V79-4 cells were plated in each well in 40 μl of 20% FBS DMEM medium. The method is referred to as (retro)transfection because the cells are added to the DNA when normally the DNA

[11] Another member of the lab, Jeff Schreifels, conducted these experiments.
[12] Another member of the lab, Patrycja Lech, conducted this experiment.
[13] Experiment and analysis conducted by Josephine Harada and Sumit Chanda at the Genomics Institute of the Novartis Foundation.

is added to the cells. After 24 hours at 37°C in 5% CO_2, 20 µl of a CSII-luciferase lentiviral vector was added to each well. Forty-eight hours later, the luminescence in each well was analyzed by adding 50 µl of the luciferase assay reagent BrightGlo (Promega; Madison, WI) to each well and luminescence was quantified with an Acquest Plate Reader (LJL Biosystems; Sunnyvale, CA). As a control, empty vector was transfected into cells (negative control) or empty vector was transfected into a 4:1 mix of 67-1 and V79-4 cells (positive control). Relative light intensity was normalized on a per-plate basis (to account for variability between 384 well plates), and genes were ranked according to light intensity relative to the mean light intensity on the plate. The top ranked 94 clones were picked from the master array and retested. As an additional control, the CSII-luciferase vector plasmid was transfected along with the cDNA using the retro(transfection) procedure. This control allowed the elimination of cDNA clones that were ranked highly simply because they increased luciferase production within the cell.

Isolation of RNA, Generation of cDNA, Sequencing

RNA was isolated utilizing a Guanidinium-Acid-Phenol Extraction protocol (Chomczynski and Sacchi, 1987). mRNA was isolated from total RNA using a Gene Elute mRNA miniprep kit (Sigma; St. Louis, MO). cDNA was synthesized using Superscript III (Invitrogen; Carlsbad, CA) as follows: 1µg of total RNA, .5 µg oligo dT, 1 µl 10 mM dNTPs, and dH_20 were combined to a total volume of 10 µl and incubated at 65 ^{o} C for 5 minutes. Samples were placed on ice and 7 µl master mix (4 µl 5x cDNA Synthesis Buffer, 2 µl .1 M DTT, and 1 µl RNase Inhibitor (Promega; Madison, WI)) was added. This mixture was incubated at 42 ^{o} C for 1 minute. One µl Superscript III

was added and the sample was incubated at 42 ^{o} C for 50 minutes. The reaction was terminated by incubation at 70 ^{o} C for 15 minutes.

To identify the sequence of MRI in the hamster cell lines PCR primers were derived from the mouse MRI sequence: mMRI-1 (F) and mMRI-1 (R). The PCR conditions to amplify MRI cDNA were 1 minute at 95^{o} C followed by 35 cycles of 95 ^{o} C for 30 s, 58 ^{o} C for 30 s, and 72 ^{o} C for 1 minute followed by a final extension of 5 minutes at 72^{o} C. cDNA amplification was carried out using Pfu polymerase (Stratagene; La Jolla, CA) and the subsequent blunt ended PCR products were cloned using Blunt Topo kit (Invitrogen; Carlsbad, CA) according to manufacturer's directions. Inserts were sequenced to determine the sequence of the transcript.

Quantifying Level of MRI Transcripts

After isolating total RNA and generating cDNA, MRI levels were quantified using qMRI-1 (F) and qMRI-1 (R). Quantitative PCR reactions using SYBR green were performed using a Biorad iCycler equipped with an optical module and BioRad SuperMix (without ROX) following the manufacturer's protocol **(**BioRad; Hercules, CA). PCR reactions contained 1 µl template, 1.5 µl each primer pair (10 mM), 12.5 µl SuperMix and 8 µl dH_20. Cycling conditions used were 95 ^{o} C for 3 min, followed by 40 cycles of 95^{o} C 30 sec, 58 ^{o} 30 sec, and 72 ^{o} 30 sec, and a final extension (5 min 72 ^{o} C) to complete all the PCR products.

Cellular localization

Immunocytochemistry was carried out as described previously (Somia et al., 1999). CSII-3x FLAG-MRI plasmid was transfected into V79-4, 67-1, A498 or MRC5 cells grown on square cover slips treated with 0.002% poly-l-lysine and placed in six well

dishes. Seventy-two hours after transfection the cells were fixed for 15 minutes with 4% formaldehyde in PBST (0.5% Tween 20 in 1X PBS). The cover slips were then washed 3X with PBST. Mouse anti-FLAG antibody (Stratagene; La Jolla, CA) was diluted 1:500 in PBST and used to probe the cover slips for 20 minutes. Cy3-labeled goat anti-mouse antibody (Jackson ImmunoResearch; West Grove, PA) was diluted 1:500 in PBST and used to probe the cover slips for 20 minutes. DAPI (1 μg/ml) (Molecular Probes; Carlsbad, CA) was added to the secondary to stain the nuclei. Images were collected on a Zeiss Apotome microscope (Zeiss; Thornwood, NY).

Identification of Effective Short Hairpin RNA (shRNA) Targeting Sequence[14]

Table 5-5: shRNAs Used

shRNA Name	Purpose	Sequence (DNA Sense Strand)
shScram	Scrambled shRNA (negative control)	GGGTATCCTATGTTGTGTGCT
shFF	target firefly luciferase gene (positive control)	TCCAATTCAGCGGGAGCCACC
sh353	target MRI	TGAGGCTGAGATAGTTGATG
sh623	target MRI	TGCTGAGATATGTCTGGGAGATGT

Two micrograms of an expression plasmid for 3x FLAG MRI, 10 μg of a retroviral vector or vector containing a U6 shRNA expression cassette, and 2 μg of pRK5-EGFP were transfected into 293T cells using established transfection protocols (Chen and Okayama, 1987). Forty-eight hours after transfection, MRI expression (using an anti-FLAG antibody M2; Sigma, St. Louis, MO) and EGFP expression (anti-EGFP antibody; Clontech, Mountain View, CA) were determined by immunoblot analysis as described above.

[14] Another member of the lab, Bryan Nikolai, conducted this experiment.

Reduction of MRI in U87 Cells[15]

U87 cells were infected with retroviral vectors transducing U6-directed shRNA targeting sequences (Rubinson et al., 2003). The retroviral vectors encoded either shScram, shFF (Paddison et al., 2002), sh353, or sh623. After infection, cells were passaged once, and 10^5 cells were infected with HIV-1 luciferase vectors and assayed as described above.

[15] Another member of the lab, Patrycja Lech, conducted this experiment.

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