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HIV-2 and SIV Vector Systems

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Abstract

Lentiviral vectors have received much attention in recent years due to their ability to efficiently transduce non-dividing cells. Of the lentiviruses HIV-2 and SIV offer several unique benefits as the basis for lentiviral vector design. HIV-1, HIV-2 and SIV remain the only known primate lentiviruses, and consequently are among the most extensively studied viruses known. Substantial effort has been devoted towards identifying the pathogenic determinants of the primate lentiviruses and towards understanding their replication within primates. Of the primate lentiviruses, the pathogenicity and rates of transmission of HIV-2 and SIV fall far below that of HIV-1, potentially providing vectors based upon HIV-2/SIV with a greater degree of biosafety. Last, and perhaps most importantly, HIV-2 and SIV are viruses which may be studied within non-human primate models susceptible to AIDS-like disease, making vectors based upon these viruses accessible to substantial preclinical evaluation. We approach this Chapter presenting information regarding the basic biology of HIV-2 and SIV and conclude by pointing to how unique features of HIV-2 and SIV are well suited to vector design, hoping to leave the reader with a greater appreciation of the potential these viruses offer within the field of gene transfer applications.

Introduction

Replication-defective retroviral vectors have traditionally been a preferred vehicle for gene transfer due to their ability to permanently integrate within the chromosome and establish stable gene expression within transduced populations. Further, the "simple" retroviral genome and its replication cycle have been extensively characterized enabling investigators to refine retroviral vectors and reduce the risk of an immune response against transduced cells by eliminating all non-essential viral elements. The absence of non-essential viral elements is likely to be a necessary feature within vectors in order to achieve sustained in vivo gene expression. Clinical use of simple retroviral vectors has been severely limited, however, due to their inability to efficiently transduce the quiescent and post-mitotic cells which are among the most desirable targets for gene therapy. The general basis for this limitation has been elucidated. Integration of the "simple" retroviral genome first requires mitotic dissolution of the nuclear membrane in order to provide the retroviral pre-integration complex (PIC) with access to cellular chromatin.[1-3] Thus, in the absence of cell division retroviral infection is blocked prior to integration.

Lentiviruses, in contrast, efficiently infect non-dividing cells during the normal course of their replication. As discussed in detail in Chapter 3, the lentiviral capacity to infect non-dividing cells may result from a direct interaction between the viral PIC and cellular nuclear

import machinery, resulting in transportation of the lentiviral PIC across the nuclear envelope where integration of proviral DNA may occur.[4] Viral components within the PIC which contribute to nuclear import may include the viral Gag matrix, integrase, and Vpr or Vpx, although the latter accessory gene products are reported to use non-classical import pathways.[5-9] The extent to which each of these components contributes to nuclear import is incompletely defined, although their cumulative effect provides the primate lentiviruses with a functional redundancy that helps insure the completion of an essential step in its replication cycle.

Initially, the potential benefits lentiviruses were thought to offer in gene transfer inspired many investigators to develop vectors and stable packaging lines based upon HIV-1. These initial studies confirmed that transduction of non-dividing cells by lentiviral vectors was feasible, although the titers which were obtained fell significantly below a clinically useful range.[10-12] This problem was in part addressed in work presented by Naldini et al describing the development of a replication-defective HIV-1 vector pseudotyped with VSV-G.[13] The vector was shown to retain the lentivirus-specific ability to infect non-dividing cells, and achieved efficient gene delivery both in vitro and by direct injection into the adult rat brain in vivo. Since these findings, a flurry of effort has been devoted to the design and characterization of lentiviral vectors.

Of the lentiviruses considered for vector design, two promising candidates are HIV-2 and SIV. HIV-2 and SIV, being primate lentiviruses, are among the most thoroughly studied and well characterized lentiviruses currently known. Extensive biological characterization of HIV-2/SIV and long term prospective studies of associated disease provide a substantial body of information to draw upon in order to assess vector behavior within a patient. This contrasts with the non-primate lentiviruses about which far less is known in terms of basic biology or their potential behavior within primates. Like all lentiviruses, both efficiently transduce a variety of non-dividing cell types. HIV-2 and SIV, however, possess features making them uniquely well suited to vector development. Among these features are a diminished pathogenic potential when compared to HIV-1, an amenability to study in primate models, and separation of the accessory gene product-associated cell cycle arrest and nuclear import functions. Each of these characteristics offer potential advantages over other lentiviral vectors and will be discussed in detail.

Our goal in this Chapter is to present current information addressing the basic biology, viral dynamics and genome organization of HIV-2/SIV as this information pertains to vector development. Because basic lentiviral replication and the design of HIV-1 vectors are discussed elsewhere within this volume, we focus in this Chapter upon areas in which the primate lentiviruses contrast with one another. Despite a variety of similarities among the primate lentiviruses, distinctions do exist. We approach this Chapter intending to address these distinctions, concluding with sections addressing the development of HIV-2/SIV vectors and potential future modifications which might be introduced to improve these vectors.

Classification and Distribution

Although a human virus, HIV-2 displays greater sequence homology with SIV than HIV-1. Sequence relatedness between HIV-2 and SIV is approximately 75%, whereas both viruses display less than 50% homology to HIV-1.[14,15] Based upon genetic diversity, six distinct subtypes (A-F) of HIV-2 have been classified.[16,17] All are found predominantly within West African countries, although infrequent cases are reported in the Americas, Europe and India. The majority of isolates characterized to date belong to subtype A, isolates of which have been obtained from diverse regions across West Africa. Subtype B predominates in Ghana and Cote d' Ivoire and constitutes the majority of HIV-2 isolates falling outside of subtype A. Rare isolates of subtypes C-F have been described in Sierra Leone and Liberia. To date, subtypes C-F have been isolated only from asymptomatic individuals, whereas the bulk of HIV-2 associated cases of AIDS which have been reported are caused by subtype A. It is tempting to speculate that this may reflect a differential virulence among the viral subtypes. Unfortunately, the

infrequent isolation of subtypes C-F and the absence of long-term prospective studies comparing subtype-specific differences makes it impossible to evaluate the statistical significance of these observations.

Nomenclature for the SIV subtypes is based upon the primate species from which prototypic viral strains representative of the subtype was isolated. Five distinct lentiviruses have been obtained from non-human primates native to Africa, including chimpanzees (SIV_{CPZ}), sooty mangabees (SIV_{SM}), african green monkeys (SIV_{AGM}), mandrills (SIV_{MND}), and sykes (SIV_{SYK}). Each of the subtypes is endemic to the primate species for which it was named and shows no evidence of disease causation within its natural host.[18]

Other designations commonly encountered in the literature include SIV_{MAC}, SIV_{MNE} and SIV_{STM}, named for SIV strains isolated at primate research centers from rhesus macaques, nemestrina macaques and stump-tailed macaques, respectively. Each of these viral strains causes a fatal AIDS-like disease in macaques. Due to the absence of evidence for SIV infection of macaques in their native Asian habitat and given the close genetic relatedness of these viral strains to SIV_{SM}, it is thought that SIV is not a virus endogenous to this species. Macaques are instead thought to have become inadvertently infected by experimental inoculation with fluids obtained from other primate species, or by co-housing of macaques with other species in research facilities.

Extensive genetic diversity is found within all of the HIV-2/SIV subtypes. As with all lentiviruses, the principal driving force to diversification is the viral mutation rate, although viral strains postulated to have arisen by means of recombination between distinct viral subtypes within the same individual have been reported. Phylogenetic analysis of the HIV and SIV *pol* sequences indicates SIV_{AGM}, SIV_{MND} and SIV_{SYK} belong to discrete lineages. HIV-1 clusters with SIV_{CPZ} in a separate lineage, whereas HIV-2 clusters with SIV_{SM}. Despite substantial sequence variation within the lentiviral subtypes, each of these lineages are roughly equidistant and show approximately 40% divergence from one another. These observations have led some investigators to speculate HIV-1 and HIV-2 may have originated through zoonotic transmission from non-human primates to humans.

Pathology and Viral Replication

Viral Transmission

The basic biology of HIV-2 and SIV, in some ways, closely resembles that of HIV-1. All three of the primate lentiviruses display common routes of transmission, cellular tropisms, long and variable incubation periods, viral replication kinetics and persistence of replication despite strong humoral and cellular immune response within the host.[19] Each spreads through exchange of bodily fluids including blood, blood products, saliva, semen, vaginal secretions and milk. Trans-placental transmission of HIV/SIV can occur, although perinatal transmission of HIV-2 occurs far less efficiently than for HIV-1.[20] Initial infection is mediated by binding of the viral surface glycoprotein gp120 to a cellular CD4 molecule which serves as the primary receptor for all of the primate lentiviruses.[21,22] Early attempts to determine if expression of the CD4 receptor was sufficient to confer susceptibility to infection to non-permisive cell types found that, although CD4 facilitates viral binding at the cell surface, it is insufficient to permit viral fusion with the cell membrane. Further, primary viral isolates display variable tropism for $CD4^+$ lymphocytes.

Together, these findings implied the necessary existence of other cellular co-factors mediating viral binding and cell fusion. After substantial effort, the chemokine receptors CCR5 and CXCR4 were identified as the principal lentiviral co-receptors.[23,24] Several studies have since shown that the CD4-glycoprotein complex, formed by viral binding to the CD4+ molecule, interacts with its chemokine coreceptor to initiate the fusion process. Conversely, viral entry

may be inhibited in the presence of RANTES, MIP-1α and MIP-1β, the ligands for CCR5, or SDF-1, the ligand for CXCR4, depending upon the biological phenotype of the viral strain.

Viral Phenotype and Coreceptor Use

The biological phenotype of primate lentiviruses, though, evolves during the course of infection. Forces driving this change include the viruses intrinsically rapid mutation rate and immunological pressures selecting for under-represented members of the lentiviral quasi-species found within the individual at any given moment. As discussed in Chapter 2, two general viral phenotypes have been described: macrophage tropic (M-tropic) and T lymphotropic (T-tropic). Of the two principal chemokine receptors, CCR5 functions as the predominant coreceptor for M-tropic HIV-1 strains, whereas CXCR4 functions as the predominant coreceptor for T-tropic HIV-1 strains. Unlike HIV-1 and HIV-2, it should be noted that SIV uses CCR5 but not CXCR4 at all stages of infection.[25] Since the initial discovery that CCR5 and CXCR4 act as the principal lentiviral coreceptors, however, at least 14 alternate lentiviral coreceptors have been identified.

In the natural progression of HIV-1 infection, a discrete series of events relating viral phenotype and chemokine receptor usage to disease prognosis has been defined. Generally, initial infection by the primate lentiviruses occurs by means of M-tropic viral strains. Supporting this claim is the fact that individuals homozygous for the CCR5Δ32/Δ32 allele are highly resistant to HIV-1 infection. Longitudinal studies of HIV-1 infection, however, report a shift towards principal usage of the CXCR4 coreceptor and more promiscuous use of alternate coreceptors late in the course of disease progression. Principal use of CXCR4 and/or promiscuous use of alternate coreceptors by HIV-1 directly relates to the appearance of syncytium-inducing (SI) T-tropic strains and correlates poorly with disease prognosis and survival.[26]

In stark contrast, many primary isolates and molecular clones of HIV-2/SIV use alternate coreceptors with efficiencies comparable to CCR5 or CXCR4.[27-29] Based upon data acquired using cell-free infectivity assays, some strains of HIV-2 appear capable of using alternate chemokine receptors even in the absence of CD4. Of note, broad coreceptor usage in HIV-2, unlike HIV-1, shows no correlation what-so-ever with syncytium-inducing capabilities. For HIV-1, promiscuous coreceptor usage has been hypothesized to either contribute to or to reflect enhanced cytopathicity. This does not hold true for HIV-2, for which disease progression is substantially slower despite promiscuous coreceptor usage at all stages of infection.

Viral Pathology

Although HIV-2 and SIV are far less pathogenic than HIV-1, they display common elements during the acute phase of infection.[18,19] Primary infection is followed by a burst of viral replication and an acute illness characterized by mononucleosis-like symptoms. Within 2 months of infection, a significant decline in $CD4^+$ T lymphocyte levels is detected in the peripheral blood. Typically, $CD4^+$ lymphocyte levels rebound, although rarely to pre-infection levels. Specific antiviral responses involving both the humoral and cellular arms of the immune system are detected approximately 1 month after infection. With the mounting of an immune response, the infected individual generally enters an asymptomatic phase during which time few, if any, disease symptoms manifest. Recent studies indicate viral replication continues to occur at a rapid rate during clinical latency, with the production of 10^9-10^{10} virions per day and the daily turnover of up to 10^9 infected $CD4^+$ T lymphocytes.[30,31] Without intervention, in the case of HIV-1, the $CD4^+$ T lymphocyte count steadily declines during the course of infection, eventually falling below 200-500/μL at which point disease symptoms become manifest and clinical AIDS ensues.

In contrast, the asymptomatic phase of HIV-2 infection is substantially longer and $CD4^+$ T lymphocyte depletion is far less pronounced. Similarly, morbidity and mortality associated

with HIV-2 is significantly lower than that of HIV-1. In the most comprehensive prospective study yet performed to compare the rates of disease progression between HIV-1 and HIV-2 infected individuals, a 67% probability of AIDS-free survival 5 years after seroconversion was reported for the HIV-1 cohort.[32] In contrast, the probability of survival over the same time period within the HIV-2 cohort was 100%. Related cross-sectional studies confirm reduced pathogenicity associated with HIV-2 as measured by a variety of indices, including $CD4^+$ counts, $CD8^+$ counts, and the $CD4^+$:$CD8^+$ ratio.[33,34] Similarly, african green monkeys and sooty mangabeys fail to develop disease despite persistent, life-long infection with SIV. In addition to its reduced pathogenicity, HIV-2 is reported to be less transmissible than HIV-1. Consistent with this finding, several investigators report observing shifts in the prevalence of HIV-1 and HIV-2 among commercial sex workers. Findings in Bamako, Mali show an increase in HIV-1 prevalence (from 10 to 35.8%) and a concurrent decrease in HIV-2 prevalence (from 15 to 3.9%) between the years 1987 and 1995.[16] Similar reports from countries in which HIV-2 is the predominant virus indicate the rate of HIV-1 infection to be increasing while HIV-2 prevalence declines. Viral and host factors contributing to disease progression in HIV infection have been the subject of extensive review.[19] Why these same factors fail to promote disease in HIV-2/SIV infection, or do so at drastically reduced rates, remains unclear at this time. Based upon these observations, however, it is possible that vectors based upon HIV-2/SIV may offer a substantial advantage over HIV-1 vectors in terms of biosafety. Efforts to address these questions will undoubtably yield insights which contribute to the development of safer lentiviral vectors.

Animal Models

One of the greatest benefits to use of HIV-2/SIV vectors over other lentiviral vectors is their amenability to study in primate models for disease. Although some strains of HIV can establish persistent infection in chimpanzees, the animals fail to exhibit disease symptoms. Further, other factors prohibit the wide use of chimpanzees in animal studies. Several strains of HIV-2/SIV, however, establish persistent infection in baboons and macaques and display varying degrees of pathogenicity. Highly pathogenic strains of HIV-2/SIV produce an AIDS-like disease within macaques which culminates in death within a matter of months.[35-38] Consequently, the biosafety of HIV-2/SIV vectors may be assessed within primates prior to clinical studies.

Another concern with regard to lentiviral vectors is the potential for vector mobilization, a process by which transfer vector RNAs are encapsidated by replication competent lentivirus present within the same organism and subsequently spread to additional tissues. In many situations vector mobilization may prove beneficial, although this remains to be evaluated. This process, and any potential hazards it may represent, may be studied using HIV-2/SIV models in primates, providing HIV-2/SIV vectors with an advantage currently unavailable to other lentiviruses. Thus, in terms of preclinical evaluation of vector biosafety, HIV-2 and SIV offer a number of unique characteristics over other lentiviral vectors.

Genome Organization and Regulation

Infectious particles of HIV-2 and SIV contain identical copies of an approximately 9 kb long single-stranded RNA genome (Fig. 1). Upon infection, a virally encoded reverse transcriptase converts this RNA genome into a linear double-stranded DNA in which identical copies of the viral long terminal repeat (LTR) flank viral genes. This DNA copy of the viral genome is subsequently integrated into the host chromosome by the virally encoded integrase. Once integrated, viral transcripts are expressed from the viral LTR using the cellular RNA Polymerase II pathway, leading to the expression of viral proteins and the accumulation of viral genomic RNAs required for viral replication.

Lentiviral gene expression is biphasic, showing a temporal shift in the pattern of gene expression during early and late stages of viral replication. The early stage of viral replication is

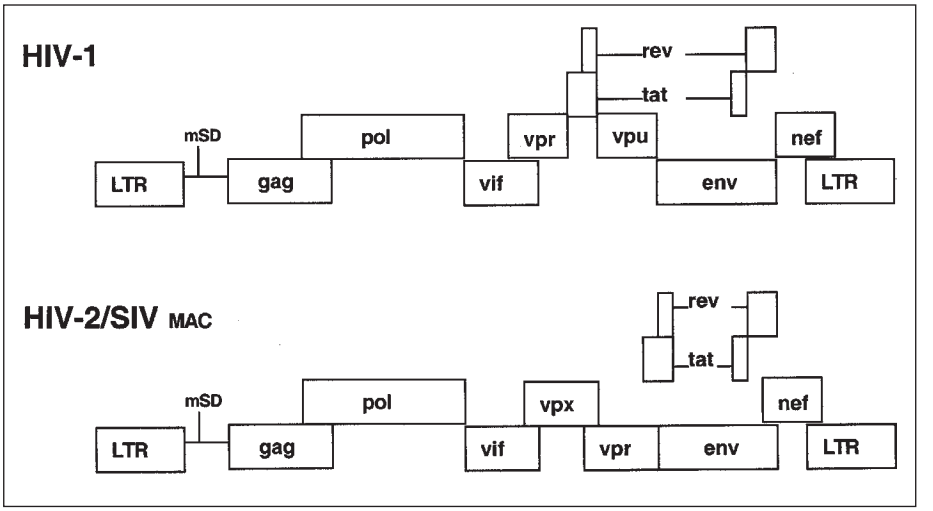

Fig. 1 Genomic organization of HIV-1 and HIV-2/SIV_{MAC}. The relative locations of the structural, regulatory, and accessory genes are indicated, MSD, major splice donor.

characterized by the appearance of multiply spliced transcripts encoding the *tat, rev* and *nef* genes. Interaction of Rev with the RRE (see below) results in expression of the late gene products. Included among these are the structural genes *gag*, *pol* and *env*, and the accessory genes *vif, vpr* and *vpu* or *vpx*.

Because many features of the viral structural, regulatory and accessory gene products are fundamentally common to all the primate lentiviruses and have been discussed elsewhere within this volume, they will only be addressed in a cursory fashion here. We will instead focus upon the relevance of these features in terms of lentiviral gene expression and/or uniqueness to HIV-2/SIV.

Structural Genes

As discussed in Chapter 2, the structural genes *gag*, *pol* and *env* comprise the basic core of all retroviral genomes. Comparison of the HIV-1, HIV-2 and SIV structural gene sequences reveals substantial sequence variation. However, the function played by these elements remains relatively constant. One noteworthy exception involves the role of the V3 loop of gp120, also referred to as the principal neutralizing determinant. The V3 loop is among the most variable regions within HIV-1, and synthetic peptides based upon V3 elicit potent type-specific neutralizing antibodies within animals. The frequency of nonsynonomous versus synonomous mutations contributing to variability within this region in HIV-1 suggests the presence of strong selective pressures driving mutation. In contrast, the V3 region of SIV is relatively conserved and fails to elicit type-specific neutralizing antibodies, whereas the V3 region of some but not all HIV-2 strains elicit neutralizing antibodies. Greater variability is instead seen in SIV within the V1, V2 and V4 regions rather than V3. Attempts to generate a chimeric virus replacing the V3 loop of SIV_{MM239} with that of $HIV\text{-}1_{MN}$ failed to produce replication competent virus, although a comparable $HIV\text{-}2_{KR}$/HIV-1 V3 chimera was capable of replication.[39] These observations suggest that tolerance for variation within this region and presumably the role played by V3 in HIV-1, HIV-2 and SIV replication significantly differ. Additional aspects of the structural genes common to all lentiviruses have been reviewed.[40]

Regulatory genes

As discussed in Chapter 2, the primate lentiviruses encode two regulatory proteins, Tat and Rev, which play essential roles in viral replication. Tat functions as a *trans*-activator of transcription and strongly elevates viral RNA abundance, acting at the levels of transcription initiation and transcription elongation. Rev acts post-transcriptionally, regulating the splicing of viral transcripts and the transport of unspliced or partially spliced transcripts to the cytoplasm. Function of both the regulatory proteins requires their interaction with *cis*-acting RNA elements. Tat requires TAR, a RNA element which forms stem-loop structures and is found at the 5' end of all viral transcripts. A complex RNA structure located within the *env* gene designated the Rev-responsive element (RRE) is required for function of Rev.

Tat

Tat is an approximately 16 kD protein encoded by two exons within the lentiviral genome. Slight variation in the molecular weight of HIV-1 Tat has been described between viral isolates (86 to 101 amino acids), although truncated 58-72 amino acid forms encoded by the first exon are functional. HIV-2 Tat, in contrast, is composed of 130 amino acids and, outside of conserved cysteine-rich and arginine-rich domains, displays little homology with HIV-1 Tat.[41] Also, unlike HIV-1 Tat, approximately 20% of the amino terminus and an additional 30% of the carboxy terminus of HIV-2 Tat is dispensable for function. Domains reported to be essential for the function of HIV-2 Tat include a cysteine-rich domain thought to comprise part of the protein activation domain, and an arginine-rich domain thought to mediate Tat:TAR binding. The HIV-1 TAR element is approximately 60 nucleotides in length and forms a single, stable RNA stem-loop containing a small pyrimidine-rich bulge which is essential to recognition and binding by Tat. The HIV-2/SIV_{MAC} TAR sequence is, in contrast, approximately 120 nucleotides in length. Models of the secondary structure within this region indicate the presence of duplicate stem loops, both of which contribute to HIV-2 Tat-TAR interaction. Of note, HIV-1 and HIV-2/SIV Tat proteins display non-reciporical complementarity.[42] The HIV-2 *trans*-activates its own LTR far more efficiently than it does the HIV-1 LTR. The HIV-1 Tat, in contrast, efficiently *trans*-activates either LTR with comparable efficiency. For all of the primate lentiviruses, however, the abundance of viral transcripts is elevated by several orders of magnitude in response to *trans*-activation by Tat.

Rev

Rev is an approximately 19 kD phosphoprotein encoded by two exons within the lentiviral genome and is expressed early in the viral replication cycle. Functionally, the protein regulates a shift in the biphasic pattern in lentiviral gene expression. Early in viral replication, only multiply spliced transcripts (encoding Tat, Rev and Nef) are detected. In the later stages of viral replication Rev binds to the *cis*-acting RRE found within unspliced and partially spliced transcripts (encoding viral structural proteins), promoting their transport, stability, and translation.

Domains playing a role in Rev's nuclear localization, RRE-specific RNA binding, oligomerization and post-trancriptional *trans*-activation of viral gene expression are highly conserved among the primate lentiviruses.[43] A basic domain situated between amino acids 35-50 contributes both to Rev's nuclear localization and binding to RRE-containing transcripts. Amino acids 18-56, extending outside of the basic domain, are implicated in Rev multimerization. A conserved leucine-rich effector domain, between amino acids 75-84, plays an essential role in nuclear export, after association with cellular factors.

Despite conservation of these functional domains within the primate lentiviruses, the HIV-1 and HIV-2/SIV Revs display non-reciporiced complementarity. HIV-2 Rev fails to function with the HIV-1 RRE, whereas HIV-1 Rev and HTLV I and II Rex proteins readily

function with the HIV-2 RRE.[44] Surprisingly, upon examination of a shorter HIV-2 RRE which more precisely defines the borders of its predicted secondary structure, the HIV-1 but not the HIV-2 Rev retained the ability to function.

The Accessory Genes

The accessory genes of HIV-1 (*vif*, *vpr*, *vpu* and *nef*) and HIV-2/SIV (*vif*, *vpr*, *vpx* and *nef*) were originally identified as a series of short open reading frames which were dispensable for viral replication within established cell lines. Since their discovery, however, a growing body of evidence suggests the accessory gene products play a central role in viral replication and pathogenesis in vivo. The function of two of these genes, *vif* and *nef*, are relatively conserved among the primate lentiviruses and so will not be discussed here. Of greater interest in terms of HIV-2/SIV vector design are *vpr* and *vpx*.

The HIV-1 Vpr is a virion-associated 14 kD protein which interacts with p6Gag. Functionally, Vpr plays two roles: inducing cell cycle arrest at the G2/M border of the cell cycle, and facilitating nuclear transport of the PIC in non-dividing cells. In contrast, these two roles are segregated within HIV-2/SIV between Vpr and Vpx, respectively.[9] The first study to show this reports that SIV_{SM} PBj1.9 proviral clones defective in *vpr* efficiently infect macrophage but fail to induce cell cycle arrest within established cell lines. Comparable PBj1.9 mutants defective in *vpx*, conversely, stimulate cell cycle arrest at the G2/M border of the cell cycle, but fail to efficiently infect primary macrophage. Of note, PBj1.9 *vpx* mutants fail to efficiently replicate in primary macrophage whether or not a simultaneous mutation is introduced into the Gag matrix NLS, indicating that HIV-2/SIV_{SM} Vpx plays a dominant role in nuclear transport of the viral PIC in monocyte-derived macrophage. This is in contrast to HIV-1, in which nuclear transport functions of the Gag matrix and Vpr overlap. With regard to vector development, these findings suggest benefits unique to HIV-2/SIV. Within HIV-2/SIV vector systems, unlike HIV-1, it is possible to eliminate the undesirable cell cycle arrest function of Vpr without sacrificing the desirable nuclear transport function of Vpx. Both features may prove to be essential in establishing optimal stable packaging lines.

LTR

Expression from the lentiviral LTR is a complex process involving the interaction of cellular basal transcription factors, virally encoded *trans*-activators, and *cis*-acting viral sequences. The LTR itself is composed of three domains, U3, R and U5, which are common to all retroviral LTRs. Transcription initiates at the U3/R boundary of the 5' LTR. Basal transcription is regulated by a core promoter region found within the U3 domain of the 5' LTR. This core promoter includes a classical TATAA box and adjacent Sp-1 binding sites. The number of Sp-1 binding sites found within U3 varies among the primate lentiviruses: the HIV-2 LTR contains four, different strains of SIV contain between two and four, and the HIV-1 LTR contains three. Studies suggest the relative spacing of Sp-1 binding sites within the LTR may influence viral *trans*-activation mediated by Tat, and that deletion of the Sp-1 binding sites reduces enhancer activity.

Also found within U3 are NF-κB enhancer elements which play a pivotal role in HIV/SIV replication.[45,46] The presence of these enhancer elements is significant, because they provide a mechanism by which other viral proteins and host mitogens and chemokines promote lentiviral gene expression. Again, the frequency of these sites varies among the primate lentiviruses. HIV-2 contains one functional NF-κB site and one non-functional site which differs slightly from the consensus GGGACTTTCC sequence. SIV contains one or two sites depending upon the viral strain examined, whereas HIV-1 has two functional NF-κB enhancer sites. The presence of a single NF-κB site, however, is sufficient to maintain wild-type enhancer activity. NF-κB-dependent *trans*-activation of the viral LTR is essential to viral

transcription in both $CD4^+$ T lymphocytes and macrophages. Cellular factors related to these processes have been the subject of several reviews.[47,48]

The R and U5 domains and adjacent regions play essential roles in reverse transcription (discussed in previous Chapters). The R domain participates in template switching during reverse transcription and contains TAR, the *cis*-acting element required for Tat-mediated *trans*-activation. Immediately downstream of the 5' U5 is an 18 nucleotide primer binding site which, in conjunction with tRNAlys, serves as the initiation site for minus strand synthesis during reverse transcription. Similarly, a poly-purine tract is found immediately upstream of the 3' U3 which serves as the intiation site for second strand synthesis during reverse transcription.

The ψ Packaging Determinant/Encapsidation Signal

The ψ sequence, defined by convention as the sequence between the major splice donor and the ATG initiation codon of *gag*, was originally described in murine oncoretroviruses as a sequence which is both necessary and sufficient for RNA encapsidation. Deletion of ψ in murine retroviruses strongly attenuates encapsidation. Conversely, attachment of ψ or ψ' (which includes ψ and a short segment of *gag*) to heterologous RNAs confers nearly wild-type levels of encapsidation.[49] The *cis*-acting sequences involved in lentiviral encapsidation, however, appear to be more complex.

In contrast to murine retroviruses, determinants of HIV encapsidation have been mapped both upstream and downstream of the major splice donor.[50-52] Because sequences within the leader region, upstream of the major splice donor, are found within both genomic and subgenomic RNAs, these findings strongly imply the existence of additional encapsidation signals which provide the viral packaging machinery with a mechanism for distinguishing between the two.

Of greater import in terms of lentiviral vector design, the ψ (or E, for encapsulation) packaging determinant of lentiviruses extends into the *gag* coding region. For this reason, a short stretch of *gag* (250-400 nucleotides) must be incorporated in optimal lentiviral transfer vectors. This effectively makes it impossible to completely separate the *cis*-acting sequences required within the transfer vector from *trans*-acting sequences which define the packaging construct because the two overlap. Two consequences result from this. The potential for homologous recombination during vector production increases, due to the presence of identical *gag* sequences within both the transfer vector and packaging construct. Also, due to the presence of *cis*-acting repressive sequences within *gag,* its incorporation into the transfer vector makes necessary the inclusion of the RRE to compensate.

Predicted secondary structure within the region is complex and involves multiple RNA stem loops which contribute to encapsidation, dimerization of the viral genome, and binding to the viral nucleocapsid. Recent analysis of the HIV-2 leader sequence and accompanying E region predicts between six and eight stem loop structures in contrast to the four stem loops ascribed to the HIV-1 leader.[53] The functional relevance of the additional stem loops within the HIV-2 leader remains unclear. Comparison of the HIV-1 and HIV-2 leader sequences is impractical due to the lack of significant homology between the two.

Vector Systems

HIV-2/SIV

Production of helper-virus free vectors based upon HIV-2 or SIV requires a split-genome design. Using this approach, viral proteins required for virion assembly and morphogenesis are expressed from packaging construct(s) stripped of *cis*-acting sequences which participate in reverse transcription, integration, and RNA encapsidation. These same *cis*-acting regions are in

turn introduced into a transfer vector. Under ideal circumstances co-expression of the packaging construct(s) and transfer vector results in encapsidation of only the transfer vector. Because the transfer vector lacks elements essential to viral replication, the vector particles produced are replication defective and are capable of only a single round of transduction. In practice, the extent to which vector prepared through use of a split genome design remains free of helper virus depends largely upon the precision with which *cis*-acting sequences required for RNA encapsidation, reverse transcription and integration are removed from the core packaging construct.

We previously described development of a replication defective VSV-G pseudotyped HIV-2 vector based upon HIV-2_{KR}.[54] HIV-2_{KR} is a molecular clone that is infectious but apathogenic in pig-tailed macaques, making vectors derived from this molecular clone amenable to study within animal models. We made use of a three plasmid transient transfection system, similar to other transiently produced lentiviral vectors. The viral structural, enzymatic and regulatory functions are provided by a packaging construct. This construct contains deletions of *cis*-acting sequences required for encapsidation, reverse transcription and integration. Also deleted is a 776 bp fragment of *env* spanning the V3 loop. VSV-G is expressed from a separate construct to allow for vector pseudotyping. As previously indicated, pseudotyping of lentiviral vectors with VSV-G broadens the vector target cell range and makes it possible to concentrate retroviral vectors by ultracentrifugation. The final construct is the transfer vector which expresses a reporter gene within the context of viral *cis*-acting sequences required for encapsidation, reverse transcription and integration.

The packaging construct we originally described expresses the viral *gag*, *pol*, *tat*, *rev*, *vif*, *vpr*, *vpx* and *nef* genes under control of the HIV-2 LTR (Fig. 2). A heterologous bovine growth hormone (BGH) polyadenylation signal replaces the 3' LTR, with the BGH p(A) being cloned precisely at the stop codon of the *nef* gene. A large portion of the *env* gene which spans the V3 loop is deleted without affecting the *tat* and *rev* coding exons or the RRE. Lastly, 61 nucleotides of the 75 nucleotide packaging signal are deleted.

We found deletion of 61 of the 75 nucleotides which comprise the HIV-2KR packaging sequence sufficient to dramatically attenuate encapsidation of the genome as measured by RNase protection assay of RNA isolated from both transfected cells and from viral pellets. More importantly, no evidence of viral replication was detected over a 6 month period when proviral clones containing this deletion (with or without an additional deletion of the 3' LTR) were transiently transfected into a highly permissive T cell line. The final packaging construct, however, permitted wild-type levels of protein expression as measured by p26 ELISA.

Several other recent studies point to the involvement of viral sequences outside of ψ in HIV and SIV encapsidation. For HIV-2, a 46 nucleotide deletion in ψ was reported to diminish genome encapsidation, but failed to abolish viral replication.[52] A more substantial 69 nucleotide deletion of ψ analyzed within this same study had catastrophic effects on LTR driven expression, reducing p26 levels to ~10% of the wild-type control value. In a related study, deletion of the HIV-2 ψ packaging region was reported to have minimal impact on genome encapsidation. This study instead identified sequences upstream of the major splice donor as the principal packaging determinant.[55] A subsequent study identified regions both upstream and downstream of the major splice donor which contribute to HIV-2 encapsidation, although no combination of deletions was found to reduce encapsidation below ~30% of the wild-type control value without drastically curtailing protein expression.[53] Sequences upstream of the major splice donor have likewise been identified as essential to efficient packaging of SIVmac genomic RNAs.[56] These apparent differences in the relative importance of ψ with regard to RNA encapsidation and viral infectivity may reflect variation attributable to genetic divergence of the individual HIV-2 molecular clones investigated.

Because most of the desirable targets for gene therapy do not express CD4 and/or the co-receptors recognized by the native primate lentiviral envelopes, we chose to pseudotype HIV-2

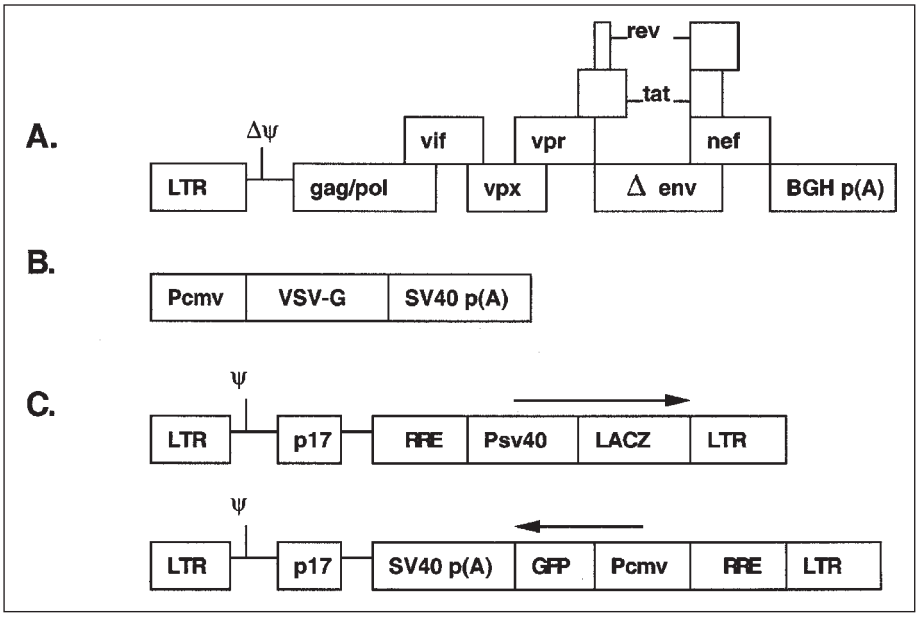

Fig. 2. A. Design of the HIV-2 packaging construct described in the text. Deletions within the packaging signal and *env* are indicated. The bovine growth hormone polyadenylation signal is positioned precisely at the translational termination codon of *nef*, replacing the 3' LTR. B. The VSV-G expression plasmid, pCMV-G. The *lacZ* and GFP transfer vectors described in the text. GFP is expressed in an antisense orientation relative to LTR expression, whereas *lacZ* expression is in a sense orientation.

with VSV-G. The receptor for VSV-G is ubiquitous. Consequently, VSV-G pseudotyping of a lentiviral vector widely broadens its tissue tropisms and target cell range. Pseudotyping with VSV-G carries with it additional advantages beyond tissue tropism. Due to the greater stability of VSV-G, pseudotyped particles may be concentrated. From the perspective of biosafety, the use of VSV-G and the absence of an intact HIV-2 *env* sequence in any of the three plasmids used makes it impossible to reconstitute the native virus through recombination.

The transfer vector we described places a *lacZ* reporter gene within the context of viral *cis*-acting sequences required for encapsidation, reverse transcription and integration. The vector contains 5' and 3' LTRs at its terminii. Viral sequences extend from the 5' LTR to include the leader sequence, ψ, and the first 373 nucleotides of *gag*. The short stretch of *gag* is followed by an approximately 1 kb *env* fragment which includes the RRE. Downstream of the RRE is an SV40 promoter-driven *lacZ* reporter placed in a sense orientation relative to the viral LTRs. A polyadenylation signal is provided for *lacZ* by the 3' LTR. Due to high background *lacZ* staining in some primary cells, we also used a CMV-IE promoter-driven GFP reporter in some instances. The GFP reporter in this vector, however, is placed in an anti-sense orientation relative to the viral LTRs and upstream of the RRE. Vector production using the three plasmid system described resulted in high titer vector (unconcentrated titer $>10^6$/mL) capable of efficiently transducing dividing and growth arrested cells, terminally differentiated neurons and primary monocyte derived macrophages.

The bio-safety of any vector, however, is significantly improved by eliminating all non-essential elements from the packaging system. This is particularly true for the primate lentiviruses for which the pathogenic determinants remain incompletely defined. For this reason, we subsequently chose to delete the accessory genes within the packaging system. Deletion of *vif, vpr,*

vpx and *nef*, individually or in combination, resulted in no significant change in vector titer on growth arrested cells or primary macrophages (unpublished data). A recent study of a comparable SIV vector found similarly that elimination of accessory genes within the packaging system had no effect on vector titer on dividing or growth arrested cells.[57] These findings are consistent with early reports of HIV-1 vectors, although one study of HIV-1 vectors found expression of *vif* and *vpr* to be essential in order to achieve efficient transduction of hepatocytes.[58] This last finding suggests that tissue specific requirements for individual accessory gene expression may prove to play an essential role in future vector design.

SIV/HIV Chimeric Vectors

One novel approach to vector design involves the development of chimeric SIV/HIV vectors. A variety of such vectors have been described, involving a three plasmid design comparable to other VSV-G pseudotyped lentiviral vectors. In the chimeric vector, however, the packaging components are provided by an SIV-based packaging construct, whereas the transfer vector is based upon HIV. Cross-packaging permits production of vector at titers comparable to that achieved with more traditional lentiviral vectors.[56] One such vector was recently shown to efficiently transdue a variety of cell types, including growth arrested cells, primary macrophages and primary mouse neurons.[59] The principal benefit SIV/HIV chimeric vectors offer is potentially greater safety. Lack of homology between the transfer vector and the packaging construct eliminates the potential for homologous recombination during vector production. However, this possible benefit must be weighed against the possibility of generating a novel virus.

Requiem and Prospectus

Although significant progress has been made in the area of lentiviral vector design, several exciting areas remain to be completely explored. Further efforts are likely to result in substantial modification of existing systems. Currently, much attention is being devoted to establishing minimal lentiviral vectors, as indicated by efforts to eliminate accessory genes from packaging systems. Now that the accessory genes have been shown to be dispensable in a number of vector systems, several investigators have turned to the regulatory genes to determine if they too might be eliminated. Particular attention has been given to the potential for replacing the RRE with the constitutive transport element (CTE) of type D retroviruses. Like RRE, CTE regulates the shuttling of viral RNAs from nucleus to cytoplasm. However, CTE makes use of an endogenous cellular pathway to perform this function, making it independent of additional viral proteins such as Rev.[60] To date, a bare handful of reports have described replacing RRE with CTE in either the packaging construct or the transfer vector.[61,62] Attempts to incorporate CTE within packaging constructs based upon HIV or SIV have unfortunately resulted in significant reduction of Gag-Pol expression and a 2-3 log reduction in vector titer. Attempts to incorporate CTE into a HIV-1 transfer vector have met with somewhat more success. One study reports obtaining titers comparable to more traditional Rev-dependent vectors, depending upon the placement of CTE relative to the 3' LTR and the presence of an additional mutation of the major splice donor within the transfer vector. It is feasible to envision, then, a lentiviral transfer vector in which the LTRs and a small fragment of *gag* are all that remain of viral sequence. From the perspective of clinical safety, a minimal vector of this sort represents an ideal.

Other recent efforts have focused on the design of self-inactivating (SIN) vectors.[57,63] SIN vectors contain a deletion of U3 within the 3' LTR of the transfer vector. Due to the template switching mechanism of reverse transcription, U3 is removed from both LTRs within the integrated vector DNA. Consequently, expression from the transfer vector within transduced cells occurs via internal promoters and not the LTR. This approach offers two distinct advantages. It prevents promoter interference within transduced cells and, it blocks expression of viral sequences, including the short stretch of *gag* included in all lentiviral vectors as part of the

encapsidation signal. A number of SIN vectors based upon HIV and SIV have been described in recent years. In all cases the titers obtained are reported to be comparable to their wild-type counterparts. SIN vectors, for these reasons, are rapidly becoming a standard element within lentiviral packaging systems.

Whereas the incorporation of SIN mutations within vector systems insures that viral sequences are not expressed within transduced cells, some investigators have begun to examine the use of tissue-specific promoters to insure transgene expression occurs only within the desired target cell population. Use of tissue-specific promoters to drive transgene expression provides lentiviral vectors with a greater degree of specificity and alleviates concern regarding the potential hazards associated with constitutive transgene expression within inappropriate tissues. The specificity engendered by such vector design is evident in one study by Miyoshi et al, in which the rhodopsin promoter was used to drive GFP expression.[64] Use of the rhodopsin promoter resulted in photoreceptor-specific expression of GFP upon direct subretinal injection of the vector, whereas CMV-driven GFP expression was detected within a variety of cells in the subretinal compartment. Similar approaches may be envisioned for other tissues.

For any vector to be clinically relevant in broad terms, however, it may be necessary to establish stable packaging lines for vector production. The transient vector preparations typically described in the literature are not readily amenable to characterization or bulk preparation, potentially limiting their applicability within a clinical setting. Stable packaging lines, conversely, may be extensively characterized and are well suited to scaling up of production. Both features may be helpful for establishing necessary quality control prior to clinical use of lentiviral vectors.

The design of forthcoming vectors is likely to incorporate aspects of each of the aforementioned features. Generation of a truly minimal packaging system will improve vector bio-safety. The use of SIN vectors within this context adds an additional layer of safety by eliminating virally directed gene expression within transduced cells. Incorporation of tissue-specific promoters within transfer vectors adds an essential level of precision to vector design, targetting transgene expression to the desired population. And, lastly, incorporation of all of these elements within a stable packaging line will allow for extensive vector characterization and bulk preparation.

Obviously the benefits of these approaches to improving vector design and/or bio-safety are not limited to HIV-2/SIV. They are, in fact, desirable features within any lentiviral vector system. Their value with regard to HIV-2 and SIV lies in improving a system that already offers unique benefits not found within other members of the lentiviral family. Again, these benefits include a drastically diminished pathogenicity relative to HIV-1, an amenability to study within primate models susceptible to disease, and a separation of nuclear import and cell cycle arrest functions. Further, being primate lentiviruses, HIV-2 and SIV are among the most extensively characterized viruses currently known, providing a basis by which to understand vector behavior within primates. Further modification of HIV-2/SIV vector design, in this context, will undoubtedly improve vector bio-safety and efficiency, bringing broad clinical application of gene transfer one step closer to reality.

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