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Using HIV-1 sequence variability to explore virus biology

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Abstract

Human immunodeficiency virus type 1 (HIV-1) only recently established an epidemic world-wide infection in the human population. The virus persists in the human host through active replication and is able to avoid clearance by the immune system. Active replication is an important component of the rapid evolutionary potential of HIV-1, a potential which manifests itself in the evolution of immune escape variants, drug resistant variants, and variants with the ability to use different cell surface coreceptors in conjunction with CD4. Multiple zoonotic introductions, compartmentalization of virus replication in the body, and genetic bottlenecks associated with sampling during transmission, antiretroviral therapy, and geographic and/or host population isolation further contribute to the range of sequences present in extant viruses. The sum of the history of all of these phenomena is reflected in HIV-1 sequence variability, and most of these phenomena are ongoing today. Here we review the use of HIV-1 sequence variability to explore its underlying biology.

Keywords: Human immunodeficiency virus (HIV); Sequence variability; Evolution

1. Introduction

HIV-1 is a member of the Lentivirus genus of the *Retroviridae* family. In the early 1980s, recognition of a new immunodeficiency disease that appeared to be communicable (CDC, 1981; Gottlieb et al., 1981; Masur et al., 1981) led to the original isolation of HIV-1 (Barre-Sinoussi et al., 1983) and the demonstration that serum samples from AIDS patients could detect the virus (Sarngadharan et al., 1984; Schupbach et al., 1984). The earliest known isolate of HIV-1 is from a

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1959 plasma sample, which suggests that the current epidemic started in this century (Zhu et al., 1998). As of the end of 2000, over 36 million people are currently infected with HIV-1 worldwide (AIDS Epidemic Update: December 2000 [http://www.us.unaids.org]).

As independent HIV-1 isolates were initially identified and characterized, it became evident that genomic heterogeneity existed among different isolates (Benn et al., 1985; Hahn et al., 1985; Wong-Staal et al., 1985). Sequence analysis confirmed these results and showed that diversity was not evenly distributed throughout the genome, but was greatest in the envelope (*env*) gene (Hahn et al., 1985; Starcich et al., 1986). Analysis of serial isolates from patients demonstrated that intrapatient isolates were more related than interpatient isolates and that intrapatient isolates exhibited evolution over time (Hahn et al., 1986). Broader groupings of HIV-1 isolates have been recognized; analysis of HIV-1 isolates from around the world has revealed eight distinct subtypes of HIV-1, which cluster together phylogenetically to form group M (for main), which is distinct from groups O (for outlier) and N (for non-M/non-O) (Robertson et al., 1999).

Like HIV-1, HIV-2 isolates exhibit genetic variability similar to what has been observed among HIV-1 isolates (Gao et al., 1994; Chen et al., 1997). HIV-2 was initially isolated from patients in West Africa who exhibited AIDS-like symptoms, but were seronegative for HIV-1 (Clavel et al., 1986). Isolation of the virus and sequencing of the genome revealed that while the genetic organization of HIV-2 was highly similar to that of HIV-1, the two viruses exhibited distant sequence similarity at the nucleotide level (Guyader et al., 1987). One difference in the genetic organization of these two viruses is that the accessory gene *vpu* is unique to HIV-1 isolates (Cohen et al., 1988), whereas *vpx* is only found in HIV-2 isolates (Henderson et al., 1988; Kappes et al., 1988).

The mechanisms that result in retroviral sequence diversity have been studied for many years (Temin, 1992), though much attention has recently been focused on HIV-1. The sequence diversity generated by these mechanisms represents the source of variants that can evade the host immune responses, that can give rise to drug-resistant strains, that can acquire altered cell tropism, and that give rise to many other biological phenomena. Collectively, these allow the persistent viral replication that ultimately weakens the host immune system and results in immunodeficiency. The goal of this review is to provide a broad overview of the use of sequence variability to study HIV-1 evolution, and to discuss the viral mechanisms and the host responses that shape this evolution.

2. Evolution of HIV in the human population

2.1. Introduction of primate lentiviruses into humans

Genetic distance analysis of the known primate lentiviruses indicates that they cluster into five distinct lineages, with HIV-1 and HIV-2 showing the highest degree of similarity with chimpanzee and sooty mangabey lentiviruses, respectively (Fig. 1). A consideration of the genetic distances within and among the five lineages has led to the view that these two human viruses originated from distinct non-human primate lentiviruses that had been evolving in their natural hosts for a long period of time before independently entering the human population (reviewed in Beer et al., 1999).

The primate reservoir that was the source of HIV-2 has been well documented as the sooty mangabey (*Cercocebus atys*) (Gao et al., 1992; Hirsch et al., 1989). Sooty mangabeys are the natural host of SIVsm and are indigenous to west Africa, which is also the epicenter of HIV-2 infection in humans. Furthermore, SIVsm is found in approximately 30% of sooty mangabeys living in the wild (Sharp et al., 1995). Phylogenetic analysis suggests that HIV-2 and SIVsm are closely related, as is SIVmac, which likely arose from the inadvertent infection of rhesus macaques with SIVsm in captivity (Fig. 1) (Hirsch et al., 1989). In a large sampling of HIV-2 and SIVsm isolates, the two lineages were found to be phylogenetically interspersed. A geographic link has been established between some phylogenetically related SIVsm and HIV-2 strains (Chen et al., 1996; Chen

et al., 1997). This suggests that there have been multiple introductions of SIVsm into the human population, representing the range of sequence diversity of the SIVsm extant population (Gao et al., 1994; Chen et al., 1997).

Recent work has indicated that the source of HIV-1 is from a subspecies of chimpanzees, *Pan troglodytes troglodytes*. Initial work with chimpanzee lentiviruses (SIVcpz) did not demonstrate a conclusive link to HIV-1. Wild-captured chimpanzees have a low seroprevalence of SIVcpz, approximately 3% (Peeters et al., 1989, 1992). Also, some SIVcpz sequences, such as SIVcpz–ANT, show less sequence similarity with HIV-1 than expected for closely related virus strains (Fig. 1) (Vanden Haesevelde et al., 1996). However, more recent work has suggested that SIVcpz strains more phylogenetically similar to HIV-1, such as SIVcpzGAB1, SIVcpzGAB2, and SIVcpzUS, were restricted to the chimpanzee sub-

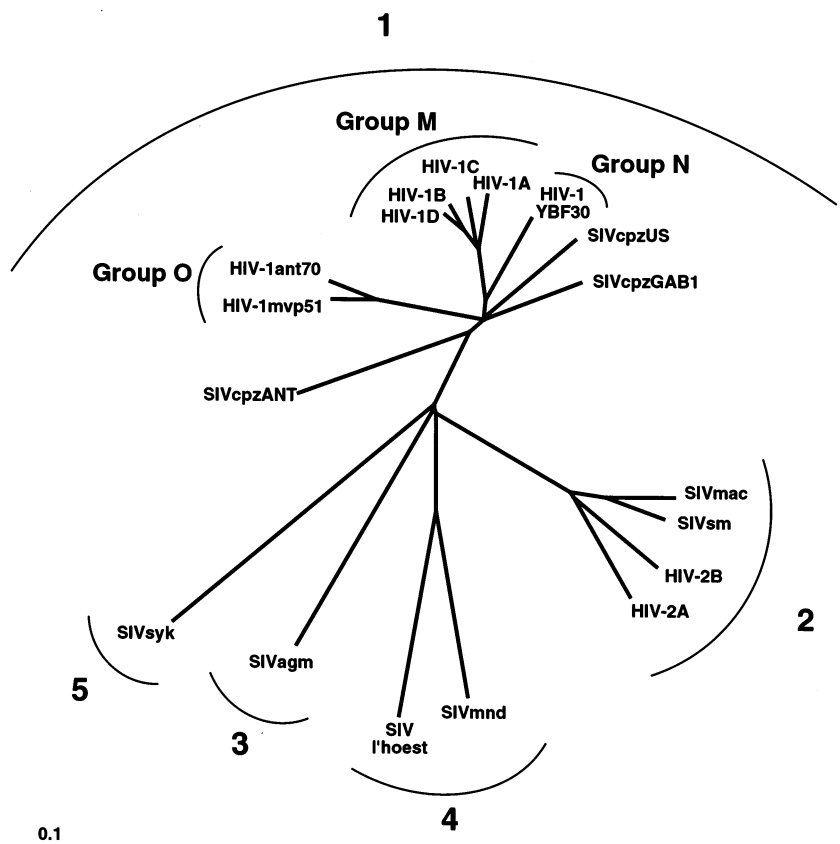

Fig. 1. Phylogenetic analysis of *pol* region from primate lentiviruses. Nucleotide sequences were aligned by using the pileup function in the GCG (Genetics Computer Group) package (University of Wisconsin, Madison). A phylogenetic tree was constructed using the neighbor-joining method [with DNAdist (Kimura 2-parameter) and Neighbor from the PHYLIP package (version 3.5c) (Felsenstein, 1993)]. The five primate lentivirus groups are numbered 1 through 5 and the three HIV-1 groups are identified. The letters A through D indicate viral clades for HIV-1 and HIV-2. The sequences were obtained from the HIV Sequence Database website (http://hiv-web.lanl.gov) under the following accession numbers: HIV-1A (U455) (M62320);, HIV-1B (HXB2) (K03455, M38432), HIV-1C (IN21068) (AF067155), HIV-1D (NDK) (M27323), HIV-1ant70 (L20587), HIV-1mvp51 (L20571), SIVcpzANT (U42720), HIV-1YBF30 (AJ006022), SIVcpzUS (AF103818), SIVcpzGAB1 (X52154), SIVmac239 (M33262), SIVsmmh4 (X14307), HIV-2A (ST) (M31113), HIV2-B (UC1) (L07625), SIVmndgb1 (M27470, X15781), SIVl'hoest (AF075269), SIVagm677a (M58410), SIVsyk173 (L06042).

species *P.t. troglodytes*, whereas SIVcpz strains that are less similar were isolated from other subspecies (Gao et al., 1999; Corbet et al., 2000). This work has provided convincing evidence that HIV-1 was introduced into humans by zoonotic transmissions from a single sub-species of chimpanzees.

A current hypothesis holds that the three most divergent groupings of HIV-1, called M, N, and O, are the result of three independent introductions of SIVcpz into the human population. Group M viruses are responsible for the majority of the human epidemic, and viral isolates in this group are divided into several subtypes or clades (Sharp et al., 1995; McCutchan et al., 1996). Group O is represented by a small number of viral isolates from Cameroon (Gürtler et al., 1996), and group N has just one known isolate, YBF30, from Cameroon (Simon et al., 1998). The genetic distances between the three groups are large, with group M and O envelope (Env) identities as low as 53% (Gao et al., 1996).

Determining when HIV-1 entered the human population has been difficult because of the many problems associated with calculating the rate of HIV-1 diversification. A key piece of evidence for the time of origin comes from the phylogenetic analysis of an early HIV-1 group M isolate from an African plasma sample stored since 1959 (Zhu et al., 1998). With its position on the group M phylogenetic tree and current evolutionary models, group M HIV-1 was probably introduced into the human population between 1915 and 1941, likely around 1931, and then continued to diversify in the human population (Korber et al., 2000b). However, there is a claim suggesting that HIV-1 was transmitted to the human population via the oral polio vaccine (OPV) in the late 1950s (Hooper, 1999). It has been suggested that some OPV stocks were grown in kidney cells prepared from SIVcpz-infected chimpanzees, which resulted in SIVcpz transmission during immunization. While it is difficult to determine exactly what happened at that time, the available evidence refutes this claim (Hillis, 2000; Korber et al., 2000a,b; Plotkin and Koprowski, 2000).

2.2. Spread and diversification in the human population

Following the introduction of group M virus into the human population, it is thought that rapid diversification and early genetic isolation of subepidemics led to the establishment of recognizable subtypes. According to criteria defined recently, eight circulating subtypes (or clades) of group M HIV-1 have been identified: A, B, C, D, F, G, H, J, and K (Robertson et al., 1999). Phylogenetic analysis of a large number of HIV-1 sequences revealed distinct sequence clusters which is the basis of the designations of distinct subtypes (Fig. 2). Within a subtype, there can exist sub-subtypes, which are distinct lineages that are very closely related to one subtype. For example, subtype F is comprised of two sub-subtypes, F1 and F2, and subtypes B and D could have been classified as sub-subtypes (Robertson et al., 1999). It appears likely that the epicenter of the group M pandemic is in Central Africa, where the earliest known group M ancestral isolate was identified and where all currently identified group M subtypes are cocirculating with high levels of genetic diversity (Zhu et al., 1998; Vidal et al., 2000).

Recombination has also contributed to the diversification of HIV-1. Retroviruses contain two copies of genomic RNA per virion, and a cell dually infected with different virus strains can produce virions containing heterodimeric RNAs. Reverse transcription of such heterodimeric RNA in a newly infected cell provides the opportunity for recombination since reverse transcriptase (RT) is able to jump between the two RNA strands during minus strand synthesis (Hu and Temin, 1990; Zhang et al., 2000). Recombinant viruses have been found involving every group and nearly every subtype of HIV-1 and HIV-2 (Kuiken et al., 1999). However, there have been no reports of recombination between HIV-1 and HIV-2, despite a large number of dual infections (Pieniazek et al., 1991; George et al., 1992). Most recombinant viruses first appeared in Africa, where all HIV-1 subtypes circulate (Robertson et al., 1998). It has been estimated that as many as 10% of all HIV-1 isolates may be intersubtype viral mosaics (Robertson et al., 1995).

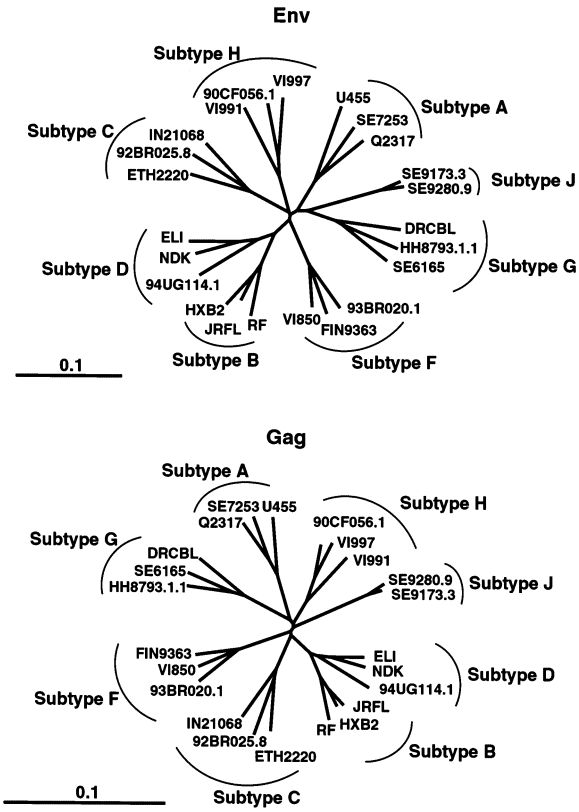

Fig. 2. Phylogenetic analysis of *env* and *gag* using representatives of the eight subtypes of HIV-1 group M. Trees were constructed as in Fig. 1. The sequences are in the HIV Sequence Database under the following accession numbers: SE7253 (AF069670), Q2317 (AF004885), JRFL (U63632), RF (M17451,M12508), ETH2220 (U46016), 92BR025.8 (U52953), ELI (K03454,X04414), 94UG114.1 (U88824), FIN9363 (AF075703), VI850 (AF077336), 93BR020.1 (AF005494), DRCBL (AF084936), SE6165 (AF061642), HH8793.1.1 (AF061640), VI991 (VI991), VI997 (VI997), 90CF056.1 (AF005496), SE9280.9 (AF082394), SE9173.3 (AF082395).

Although there are many HIV-1 recombinants, not all of them play a major role in the HIV-1 pandemic. Mosaic strains that are involved in expanding HIV-1 infections have been referred to as 'circulating recombinant forms' (CRF) (Carr et al., 1998). At present, there are eleven recognized CRFs throughout the world (HIV Sequence Database [http://hiv-web.lanl.gov]). For example, an AE recombinant is found extensively in southeast Asia, an AG recombinant in west and central Africa, and an AB recombinant in Russia (Fig. 3) (Robertson et al., 1999). It is possible that AE is not a recombinant, as some statistical tests suggest that AE is monophyletic (Anderson et al., 2000). Continuous monitoring will be necessary to establish whether these recombinants come to represent a more significant part in the HIV-1 pandemic and what role they might play in limiting vaccine efficacy.

3. HIV-1 evolution within an individual

HIV-1 evolution during chronic infection is potentiated by the large number of rounds of virus replication and the inherent error-prone nature of reverse transcription. At any given time, the virus population of an HIV-1 infected individual is heterogeneous and dynamic, with the cross-sectional diversity of the *env* population initially increasing at the earlier stages of infection, but declining toward the later stages of infection (Delwart et al., 1997; Shankarappa et al., 1999). This section focuses on the viral mechanisms that fuel HIV-1 evolution and the host characteristics that help to shape the HIV-1 population within an individual.

3.1. Viral mechanisms of HIV-1 diversity

There are two viral mechanisms that contribute to the generation of variability of HIV-1 within an individual. One is the introduction of mutations into the viral genome by the error-prone reverse transcriptase (RT), and the other is recombination between pre-existing viral populations. The rate of HIV-1 evolution, which is dependent on these two mechanisms, will also be discussed in this section.

3.1.1. Mutation rate of HIV-1

Retrovirus replication involves the use of three separate polymerase systems, each with its own distinct potential for introducing errors. In the integrated (proviral) state of viral DNA, the cellular DNA replication machinery carries out replication of the viral genetic information, presumably with high fidelity. Synthesis of viral genomic RNA by the host RNA polymerase II is

a potentially error-prone step, although at present the contribution of this step in introducing mutations is poorly understood. More often, error-prone DNA synthesis by the viral DNA polymerase, RT, is envisioned as the ultimate source of viral sequence diversity. Numerous studies have documented that retroviral polymerases are error-prone in vitro (reviewed in Preston and Dougherty, 1996). Most studies of purified RT have over-estimated the potential error rate in vivo, which is significantly lower than that observed in vitro (Varela-Echavarria et al., 1992; Mansky and Temin, 1995), but still significantly higher than the fidelity associated with host DNA synthesis.

Several different retroviruses have been examined to establish the range of error rates per round of replication. From these studies, the rate of all types of mutations combined is in the range of 0.1–1 mutation per genome per round of replication (reviewed in Preston and Dougherty, 1996). A major contribution to the propensity of RT to incorporate point mutations is the absence of an exonuclease activity that would permit proofreading of misincorporated bases, although potential rudimentary proofreading mechanisms involving pyrophosphorolysis (Arion et al., 1998) or dinucleotide polyphosphate synthesis (Meyer et al., 1998) have been uncovered.

In studies performed using HIV-1, the in vivo rate for point mutations was found to be 3.4×10^{-5} (Mansky and Temin, 1995; Mansky, 1996a). Thus a majority of HIV-1 daughter genomes are faithful copies of the parental genome, but on average between three and four out of ten will carry a new point mutation. Several factors have

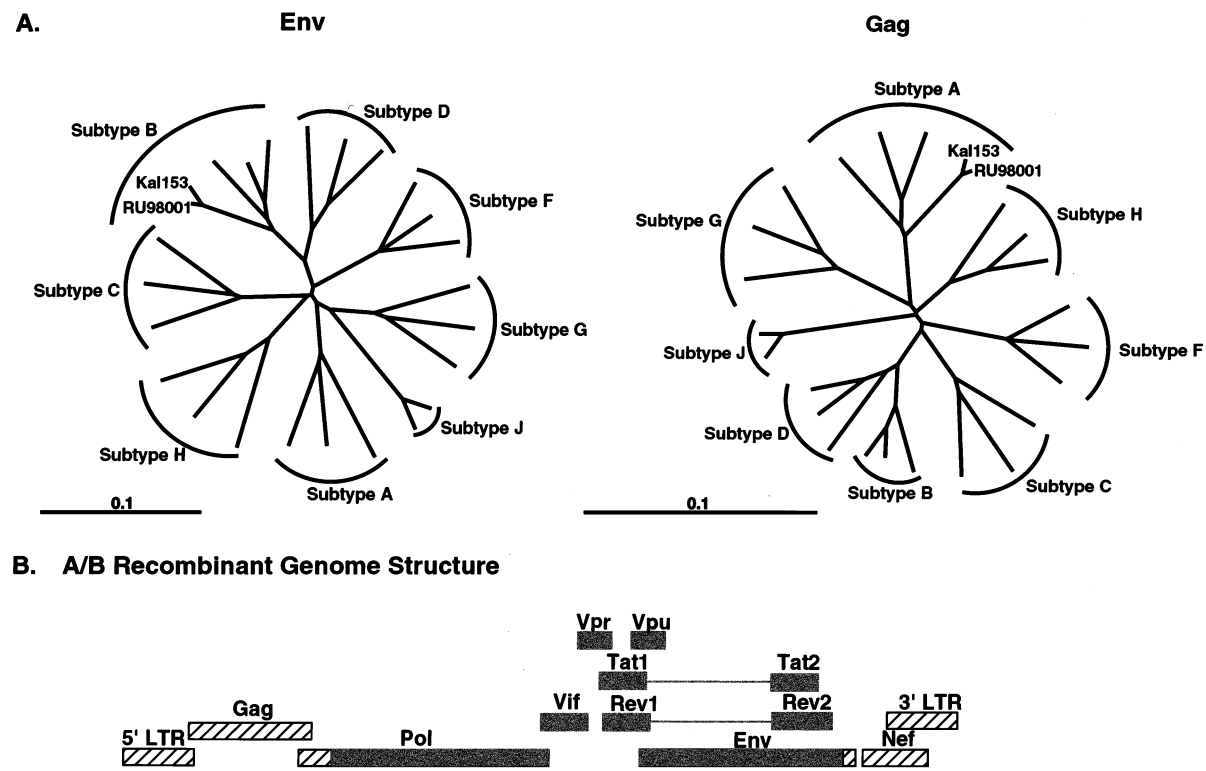

Fig. 3. (A) Differential clustering of a HIV-1 recombinant subtype in the *env* (subtype B) and *gag* (subtype A) regions. The noted sequences in the trees are A/B recombinants. Trees were constructed as in Fig. 1. The sequences came from the HIV Sequence Database: Kal153 (AF193276) and RU98001 (AF193277). (B) Schematic representation of HIV-1 A/B recombinant genome. Hatched areas have subtype A-like sequence and black areas have subtype B-like sequence. Adapted from Robertson et al. (1999).

been identified that can alter the mutation rate of HIV-1. Mutations in *vpr* increase the error rate approximately threefold but do not change the spectrum of mutations (Mansky, 1996b). The Vpr protein has been reported to bind to several cellular proteins, in particular uracil *N*-glycosylase (UNG) and the human homologue of yeast RAD23 (HHR23A), which are both involved in DNA repair (Zhao et al., 1994; Refaeli et al., 1995). The ability of Vpr to bind to UNG correlates with its ability to affect the mutation rate (Mansky et al., 2000). Hypermutation, which is the high density accumulation of a specific type of nucleotide substitution, can also contribute to the mutation spectrum (Vartanian et al., 1991; Kim et al., 1996). Several different types of hypermutation have been noted (although G to A is the most common), and each may arise under conditions of deoxynucleotide pool imbalance (Martinez et al., 1994). The presence of either RT inhibitor AZT or 3TC has been shown to increase mutation rates in vivo (Julias et al., 1997; Mansky and Bernard, 2000). Furthermore, AZT-resistant RT has an increased mutation rate (Mansky and Bernard, 2000).

The types of mutations that are introduced by RT are far from random, although an insufficient number of mutations have been characterized to understand all of the determinants of mutation bias. The majority of mutations are substitutions, with G to A transitions by far the most common. Frameshift mutations occur several fold less frequently than substitution mutations and most often represent the addition or deletion of a nucleotide in a homopolymeric run (Pathak and Temin, 1990a,b). Approximately one in 10 genomes suffers a significant internal deletion during a single round of replication (Olsen et al., 1990; Parthasarathi et al., 1995). Deletions are strongly potentiated by the presence of direct repeats (Delviks and Pathak, 1999, and others within).

3.1.2. Recombination

Recombination between related viral variants within an infected individual provides another source of variability. Interclade recombination, as described above, results in large evolutionary steps, whereas intraclade recombination results in smaller evolutionary changes among related variants. There have been reports of indirect evidence of intraclade recombination, in which different regions of the genome appear to be evolving independently (Simmonds et al., 1991; Morris et al., 1999). Demonstration of recombination in an individual infected with subtype B HIV-1 was found in a comparison of infectious molecular clones from proviral DNA (Groenink et al., 1992). Recombination can be an efficient mechanism for producing new combinations of sequences; for example, a rhesus monkey had predominantly wild type SIVmac239 only 2 weeks after being infected with two SIVmac239 strains that were attenuated in different genes and were injected into different legs (Wooley et al., 1997). We have found evidence of recombination between related variants in an individual infected with subtype B HIV-1, where recombination events in the V1/V2 region of *env* generated new sequence variants that rapidly achieved fixation, possibly the result of immune selection (McGrath and Swanstrom, unpublished results).

3.1.3. Rate of HIV-1 evolution

The observed rate of HIV-1 evolution in vivo is determined by the rate of mutation per generation (resulting from RT errors and recombination), the number of generations, and the fixation rate of mutations (through selection or genetic bottlenecks). Because the rate of fixation and number of generations are difficult to observe directly, the rate of HIV-1 evolution during human infection is usually estimated from the slope of a line that best fits a plot of nucleotide sequence divergence from an ancestral sequence versus time. Using branch lengths of a phylogenetic tree generated by maximum-likelihood methods as a measure of divergence, Korber and colleagues recently estimated the rate of evolution to be 0.24% (0.18–0.28%) substitutions per base pair per year for *env* coding sequences and 0.19% (0.09–0.27%) for *gag* (Korber et al., 2000b). However, previously published rates of divergence approaching 1% per year cannot be ruled out because of the variability of rate estimates (discussed below) (Lukashov et al., 1995; Zhang et al., 1997a; Leitner and Albert, 1999; Shankarappa et al., 1999).

One complication to determining the rate of HIV-1 divergence is that models of evolution and phylogenetic techniques used to calculate divergence typically do not take recombination into account (Korber et al., 1998). Rates of evolution have also been shown or are hypothesized to differ: (1) from one genomic region to another (Leitner and Albert, 1999); (2) among different sublineages of virus within a host (Korber et al., 1998); (3) during different stages of infection (Shankarappa et al., 1999); and (4) between hosts (Lukashov et al., 1995; Zhang et al., 1997a). In addition, estimates of the evolutionary rate depend on the alignment methods and the evolutionary model used (Korber et al., 2000b). Thus, the assignment of a rate of HIV-1 evolution is context-dependent, and generalizations must be made with care.

3.2. The HIV-1 population in an infected individual

All mechanisms described thus far result in the replication of HIV-1 as a group of closely related but non-identical sequences, a type of population that has been termed a quasispecies (Eigen, 1971; reviewed in Domingo and Holland, 1997). The behavior of such a quasispecies depends on the fraction of individual viruses that actively partake in successful replication. The following section presents different arguments pertaining to the size of the actively replicating HIV-1 population within an individual. The effect of compartmentalization of viral replication on diversity and the link between disease progression and viral diversity also will be examined.

3.2.1. Effective population size

Only a fraction of the total viral population within an individual contributes to the gene pool of the next generation of virus. The size of this fraction is referred to as the effective population size, as opposed to the total population size. The effective population size is an important determinant of the influence of stochastic versus deterministic effects in viral evolution. In a population of small effective size, the fate of viral variants can be influenced by random events, independent of their fitness compared to other variants present, i.e. stochastic behavior. In large populations undergoing many rounds of replication, the abundance of variants is not subject to random events, but is governed only by their fitness, i.e. deterministic behavior. The magnitude of a variant's selective advantage is also important in determining its fate in the total viral population. Variants with small selective advantages or neutral mutations tend to be governed by stochastic effects, whereas variants with large selective advantages behave more deterministically (Rouzine and Coffin, 1999).

The effective size of the HIV-1 population has yet to be determined conclusively. In one view there is a large HIV-1 effective population size within an individual, which may be greater than 3×10^5, the inverse of the HIV-1 mutation rate (Coffin, 1995). If this is true, every possible point mutation is generated every day, and relative fitness determines the level at which a mutant sequence persists in the viral population. The high number of productively infected cells during chronic infection, between 10^7 and 10^8 (Haase et al., 1996), and the generation of an estimated 10^{10} virions per day (Perelson et al., 1996) support this model. Further evidence for a large effective population size was recently obtained from the analysis of *pro* sequences, in which the population size was estimated to be at least 10^5 (Rouzine and Coffin, 1999).

Other observations seem more consistent with a smaller effective population size. Viral population size has been estimated from the analysis of *env* sequences to be between 1×10^3 and 2×10^3, approximately 4–5 orders of magnitude lower than the number of infected cells (Leigh Brown, 1997; Leigh Brown and Richman, 1997). The small effective population size compared to the large number of infected cells could be due to a subdivision of the total population into independent populations, frequent bottleneck events, or the production of virus particles by a large fraction of cells that do not contribute to the next round of infection. The complex antiretroviral resistance patterns that have been observed in infected individuals may lend support to the stochastic model of evolution (Leigh Brown and Richman, 1997).

The large and small effective population size models have been generally treated as mutually exclusive. However, it has been suggested that neither model alone can describe the evolution of HIV-1 under all conditions (Rouzine and Coffin, 1999). HIV-1 may behave mostly deterministically during the steady state of infection, but events such as bottlenecks during drug treatment or transmission may increase the importance of stochastic effects. At this time the effective population size remains both a critically important parameter for understanding HIV evolution and a source of ongoing discussion.

3.2.2. Compartmentalization

The HIV-1 population within an infected individual is heterogeneous, although this heterogeneity may not be evenly distributed in the body. Disparity has been found between viral sub-populations that develop in different compartments of the body, particularly in the brain, blood, genital tract, oral cavity, and lymphoid tissue. Several ideas have been presented to explain the compartmentalization phenomenon, such as physical isolation of a tissue compartment, selective migration of infected cells, distinct types of target cells, and localized selective pressures. Understanding the differences in viral evolution in distinct compartments is important for studying issues such as sexual transmission, bioavailability of antiviral drugs, and virus–host interactions.

Since the predominant mode of HIV-1 transmission is sexual, it is important to understand the biology of HIV-1 in genital secretions. There are several lines of evidence to suggest that the blood and the male genital tract are at least partially distinct compartments. First, there is at most a weak correlation between the levels of either CD4 + T cells or viral RNA load in blood and viral RNA load in semen (Liuzzi et al., 1996; Kiessling et al., 1998; Vernazza et al., 1999). Second, responses to antivirals can differ between the blood and in the male genital tract, indicating that drug bioavailability may vary between these sites (Coombs et al., 1998; Eron et al., 1998; Kashuba et al., 1999). Third, the distribution of *env* gene species in blood and male genital tract is sometimes discordant (Vernazza et al., 1994; Coombs et al., 1998; Ping et al., 2000). Last, the majority variant in blood is not always the majority variant found in semen, whether comparing DNA or RNA (Delwart et al., 1998; Eron et al., 1998; Kiessling et al., 1998).

There are similar lines of evidence that the blood and female genital tract are not a single, well mixed compartment, such as discordant viral loads (Rasheed, 1998), differential responses to nucleoside RT inhibitors (Rasheed et al., 1996), and diverse populations in both compartments (Poss et al., 1995; Overbaugh et al., 1996; Poss et al., 1998). Increased HIV-1 shedding in the genital tracts of men and women has been associated with localized inflammation, indicating that variants may be established through the migration of infected lymphocytes (Clemetson et al., 1993; Kreiss et al., 1994; Anderson et al., 1998). Understanding the selection of certain viral variants in the genital tract is a necessary part of understanding the genetic bottlenecks of infection and of HIV-1 transmission.

The brain can be considered a separate compartment for HIV-1 because of the blood–brain barrier. When brain-derived sequences have been compared to sequences found in other parts of the body they usually form a separate cluster (Steuler et al., 1992; Korber et al., 1994; Wong et al., 1997). Previous work has focused on comparing *env* sequences in the brain to *env* sequences from other compartments of the same individual, because the viral surface protein is often the determinant of virulence or tropism in different cell types (Power et al., 1994). Viruses replicating in the brain are macrophage-tropic, which is consistent with the type of infected cells found in the brain (Epstein et al., 1991; Keys et al., 1993; Korber et al., 1994; Power et al., 1994).

Virus in the oral cavity appears to be largely from the same compartment as the blood. Several studies have compared viral RNA loads in blood versus saliva, and most have found that they are positively correlated, despite technical differences between studies (Phillips et al., 1994; Liuzzi et al., 1996; Shugars et al., 2000). Analysis of V3 *env* sequences in blood and saliva at the viral RNA and proviral DNA levels have shown that the same variants usually exist in both compartments,

although there are a few instances of differences (Kakizawa et al., 1996; Freel et al., 2001). Recent evidence suggests that inflammation in the oral cavity is associated with an increase in viral loads, similar to what has been seen in the genital tract (Uccini et al., 1999; Shugars et al., 2000).

Virus isolated from the lymph nodes also appears to represent the same compartment as the blood. The majority of HIV-1 virions are produced in the lymphoid organs, and virions not trapped by the follicular dendritic cells can enter the bloodstream (reviewed in Fauci and Desrosiers, 1997). Several studies have demonstrated that variants in the blood are similar to those in lymph nodes (Ball et al., 1994; Donaldson et al., 1994). Other studies have shown similarities in variants from lymph nodes and in non-lymphoid organs such as the brain and lungs, suggesting that lymphocytes that were infected in the lymph nodes have infiltrated these organs and are the source of virus (Reddy et al., 1996; Hughes et al., 1997; Morris et al., 1999). One study looked at the distribution of proviral DNA variants across the spleen and found a non-homogenous distribution (Delassus et al., 1992). One difference has been found in the blood plasma/lymphoid cell pools of virus, specifically between the viral RNA from virions and the proviral DNA from the infected lymphocytes from the same blood sample. Typically, variants in the viral RNA at one timepoint are found in the proviral DNA at later timepoints (Simmonds et al., 1991; Ball et al., 1994), which suggests that a significant fraction of the infected lymphocytes represent a potential reservoir for archival viral sequences. Similarly, drug resistance markers show a delayed appearance in viral DNA compared to viral RNA, indicating that actively replicating sequences represent only a fraction of the total viral DNA in PBMCs (Smith et al., 1993; Lineberger et al., 1995; Wei et al., 1995).

3.2.3. Viral diversity versus progression rate

The rate of progression to AIDS within an individual can be predicted by the steady state viral load, or set point (Gupta et al., 1993; Mellors et al., 1995, 1996). Steady state is achieved when immune responses are able to partially control viral replication following primary infection (reviewed in Coffin, 1996). Higher viral loads have been shown to be associated with faster disease progression (Mellors et al., 1996), and this prediction can be made as soon as 6–12 months following seroconversion (Mellors et al., 1995; Lefrere et al., 1998).

The extent to which host factors versus viral factors determine the rate of disease progression is unknown. Several studies have focused on differences between slow and rapid progressors. Typically, infected individuals progress to AIDS within 10 years, but some individuals progress much more rapidly and some individuals remain AIDS-free for more than 10 years. Slow progressors usually have higher neutralizing antibody titers and stronger cytotoxic T-lymphocyte (CTL) responses to HIV-1 than patients with typical progression (Pantaleo et al., 1995; Montefiori et al., 1996; Betts et al., 1999). In contrast, rapid progressors typically have weak or no neutralizing antibodies toward HIV-1 (Cao et al., 1995; Liu et al., 1997).

Viral divergence and diversity also appear to correlate with the rate of disease progression. Patients with slower disease progression have higher ratios of nonsynonymous to synonymous site mutations as well as higher diversity of viral quasispecies (Lukashov et al., 1995; Wolinsky et al., 1996; Delwart et al., 1997; Liu et al., 1997). The antigenic diversity threshold model, which is based on mathematical models, attempts to explain the difference in evolution in slow and rapid progressors by suggesting that the immune system will be exhausted when the accumulation of viral variants reaches a certain threshold (Wodarz and Nowak, 1999). The threshold for each individual is different, which could explain the differences seen between rapid and slow progressors. The continuous virus adaptation model (Lukashov and Goudsmit, 1998), on the other hand, suggests that a competent immune system causes the virus to constantly evolve and adapt to the changing environment. This ability to adapt allows the virus to undergo continuous replication which leads to the depletion of CD4 + T cells and, ultimately, the development of AIDS. The former model would predict similar rates of evolution

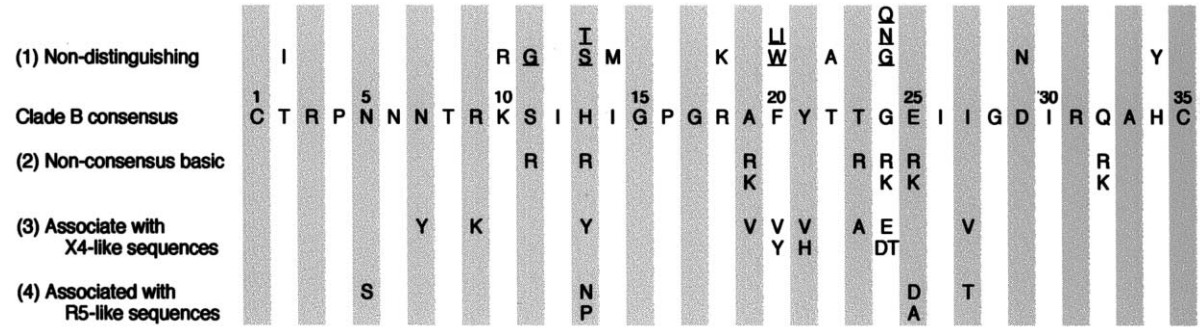

Fig. 4. Summary of sequence variability in V3. Subtype B V3 sequences were classified as SI-like based on the presence of at least one non-consensus basic substitution listed in line (2), plus a change away from an acidic amino-acid at position 25; other sequences were considered NSI-like. A statistical test was designed to assess the distribution of substitutions in the resulting two sequence sets. Frequencies of substitutions indicated in lines (3) and (4) deviated significantly from expected values, and these changes were thus classified as either SI-associated or NSI-associated, respectively. Substitutions indicated in line (1) were not significantly associated with either group, although underlined amino acids were overrepresented among SI-like V3 sequences. Adapted from Milich et al. (1997).

until the threshold is reached. This prediction is inconsistent with data comparing slow and rapid progressors (Delwart et al., 1997).

4. Selective pressures within the host

HIV-1 evolution within an individual is directed by selective pressures placed on the virus by the host that act on different parts of the virus at different times during infection. This section focuses on the interplay between pressures imposed on viral replication within the host environment and compensatory changes in viral populations.

4.1. Cell-type specificity

Generally, HIV-1 can only infect cells that express CD4 and one of two chemokine receptors, CCR5 or CXCR4 (reviewed in Moore et al., 1997; Berger et al., 1999). The preferred phenotypic classification for HIV-1 isolates is defined by this CD4-dependent chemokine receptor usage: R5 for isolates using CCR5 for entry, X4 for those using CXCR4, and R5X4 for those capable of using both coreceptors (Berger et al., 1998). Other common phenotypic classifications that generally correlate with the R5 and X4 designations, respectively, are growth rate in vitro (slow/low vs. rapid/high), cell tropism (macrophage-tropic vs. T cell line-tropic), or the ability to induce multinucleated giant cell formation in cultured T-cell lines (non-syncytium inducing/NSI vs. syncytium inducing/SI) (Asjo et al., 1986; Tersmette et al., 1988; Schuitemaker et al., 1992). Coreceptor usage does not fully explain tropism, however, since most X4 isolates cannot infect macrophages despite the expression of CXCR4 by those cells (Cheng-Mayer et al., 1997). There are also at least two mechanisms for dual-tropism: usage of either CXCR4 or CCR5 to enter either macrophages or T-cells (dual-tropic R5X4), and exclusive usage of CXCR4 to enter either of these cell types (dual-tropic X4) (Yi et al., 1999). These observations raise questions about the possible roles of post-entry factors in determining tropism.

To whatever extent growth kinetics, syncytium induction, and tropism are tied to coreceptor usage, all are determined to a large extent by sequences in the third variable loop (V3) of the HIV-1 gp120 envelope glycoprotein (Hwang et al., 1991; Cann et al., 1992; de Jong et al., 1992; Cocchi et al., 1996; Trkola et al., 1996). CXCR4 usage is most often associated with an accumulation of positively-charged amino acid substitutions in V3, particularly at positions 11 and 25 (Fig. 4) (Chesebro et al., 1992; Fouchier et al., 1992; Milich et al., 1993; Chesebro et al., 1996). In

many contexts, a change in V3 is both necessary and sufficient to produce a change in coreceptor usage (Xiao et al., 1998). However, there is some suggestion that sites outside of V3, particularly in the C4 region, vary with V3 and influence coreceptor usage by modifying either the internal stability of gp120, or coreceptor binding (Carrillo and Ratner, 1996; Milich et al., 1997).

In most patients, only R5/NSI viruses can be isolated during acute HIV infection and immediately after seroconversion (Roos et al., 1992; Zhu et al., 1993). Although occasionally X4/SI isolates can be detected early, they usually appear later in infection and are associated with progression to AIDS (Tersmette et al., 1989). It has been suggested that the presence of X4 variants may be transient, which could hinder their detection during disease progression (Shankarappa et al., 1999). In a large prospective study, Koot et al. (1993) reported that HIV-1-infected patients were over six-fold more likely to progress to AIDS within 30 months when SI isolates were detected. X4/SI detection is also predictive of rapid CD4 cell decline (Koot et al., 1993; Connor et al., 1997). The low frequency of transmission of X4 variants is unexplained and likely results from one or more of the following explanations: (i) poor representation of X4 variants in genital secretions; (ii) few target cells at mucosal surfaces; (iii) selective clearance of X4 variants during primary infection; and (iv) a predominance of CCR5-expressing activated T cells at lymphoid tissue sites that sustain the infection (Harouse et al., 1999).

The use of a V3-specific heteroduplex tracking assay (V3-HTA) (Nelson et al., 1997) has allowed the quantitative assessment of the dynamic interaction between R5 and X4 variants. In this analysis, these variants have much more complex interactions, including the persistence of R5 variants as the major species even in the presence of X4 variants, and the persistence of low-abundance variants with highly evolved V3 sequences (Nelson et al., 2000). The association of X4 variants with disease progression, and the maintenance of R5 variants throughout infection suggest that X4 viruses are either a cause of, or evolve in response to, progressive immunosuppression, while R5 variants make up the reservoir responsible for persistent infection (Schuitemaker et al., 1992).

4.2. Transmission

Transmission of HIV-1 is typically accompanied by a loss of both genotypic and phenotypic viral heterogeneity (Wolfs et al., 1992). Patients with longstanding HIV infections contain complex viral populations that display a range of phenotypes, whereas recent seroconverters contain a more genotypically homogenous virus that is uniformly R5/NSI (Zhu et al., 1993; Shankarappa et al., 1998). X4/SI variants are rarely sampled in recipients even when detected in the transmitter (Roos et al., 1992). Studies of epidemiologically related patients have shown that the predominant species detected in recently infected recipients are usually sampled as minor variants present in the blood of the transmitter (Wolinsky et al., 1992; Zhu et al., 1993). Viral heterogeneity early in infection may be influenced by the sex of the recipient; in one study of recently infected Africans who had acquired HIV-1 infections via heterosexual contact, women were found to harbor multiple viral variants, while the virus in men was more homogeneous (Long et al., 2000).

The purifying effect of transmission is most pronounced in *env*, particularly in the V3 region (Pang et al., 1992; Zhu et al., 1993). Soon after infection, V3 is less variable than other regions of the genome, such as V4-V5 of gp120 (Wolinsky et al., 1992) and p17/MA (Zhang et al., 1993), while in the chronically infected individual, gp120 and the V3 loop are of above-average diversity compared to the rest of the genome. In addition, high ratios of the frequency of synonymous to nonsynonymous mutations (Ks/Ka) in V3 relative to V4–V5 soon after infection indicate the suppression of variants bearing coding changes in V3 during initial infection (Wolinsky et al., 1992). During chronic infection, there are low Ks/Ka ratios for V3, indicating selection for diversification in that region (Seibert et al., 1995; Wolinsky et al., 1996; Zhang et al., 1997a).

After transmission, both the timing and nature of the selective pressures responsible for the re-

duction in sequence variability remain poorly defined. Selection likely occurs very soon after transmission, but before seroconversion (Zhang et al., 1993). Strong early selection on V3 accompanied by recombination among members of an initially diverse population is consistent with the observed homogeneity of V3 and heterogeneity of other genomic regions (Zhang et al., 1993). The nature of this selective pressure remains controversial. It has been suggested that V3 loops of R5 viruses are more constrained in sequence than X4 viruses by the functional requirements of the interaction with the coreceptor (Sato et al., 1999). Selective replication of R5 viruses might also be a result of tropism requirements, since R5 variants have been shown to preferentially replicate in sub-optimally activated T-cells (Vicenzi et al., 1999). This in vitro observation was not borne out in HIV-infected patients, however, where naïve T cells contained culturable virus and proviral DNA representing both X4 and R5 variants at all stages of disease (Ostrowski et al., 1999).

4.3. Immune responses

Anti-HIV-1 immune responses drive viral evolution by selecting variants with reduced sensitivity to neutralizing antibodies and CTLs. The emergence of CD8+ CTL directed against HIV-1 epitopes coincides with a significant drop in initial viremia during primary infection, with most individuals having a measurable anti-HIV-1 CTL response at the time that they present with symptoms of acute HIV-1 infection (Borrow et al., 1994; Koup et al., 1994). The initial CTL response is important for containing primary infection because a lack of a sustained CTL response against several HIV-1 epitopes results in rapid progression to AIDS (Hay et al., 1999). In one study, a broader CTL response against HIV-1 was associated with a slower rate of CD4+ T cell loss (Pantaleo et al., 1997). Anti-HIV-1 memory CTLs are typically maintained at high levels during asymptomatic infection, and the loss of this CTL response is correlated with progression to AIDS (Rinaldo et al., 1995). Similarly, ablation of CD8+ cells in the macaque/SIV model leads to dramatic increases in viral load (Jin et al., 1999; Schmitz et al., 1999).

The best evidence of the potency of the CTL response, as well as a measurement of the selective pressure exerted on HIV-1 by the CTL response, is the emergence of CTL escape mutants. Escape mutants were first described in chronically infected individuals who were followed over several years (Phillips et al., 1991). In two separate studies of individuals with primary infection, CTL escape mutants were found just 22–30 days after the onset of symptoms of acute infection (Borrow et al., 1997; Price et al., 1997). In both studies, the escape mutants rapidly became the dominant variants in vivo, and these individuals had rapid CD4+ T cell losses indicative of rapid disease progression. In one SIV-infected macaque study, it was demonstrated that CTL responses specific for Tat, a viral protein expressed early in the life cycle, selected for viral escape variants (Allen et al., 2000). In long-term asymptomatic HIV-1-infected individuals and in SIV-infected rhesus macaques, the appearance of CTL escape mutants is associated with disease progression (Goulder et al., 1997; Evans et al., 1999). Correlations with viral load for both the initial anti-HIV-1 CTL response and the emergence of CTL escape mutants suggest that the effectiveness of CTLs against HIV-1 may determine the length of the asymptomatic period of infection.

The neutralizing antibody response takes longer to develop than HIV-1-specific CTL responses. Neutralizing antibody responses against autologous HIV-1 strains have been detected as soon as 5 weeks after the onset of primary infection symptoms, although they are typically seen any time up to 1 year after seroconversion (Arendrup et al., 1992; Pilgrim et al., 1997). Long-term non-progressors tend to have broader neutralizing antibody responses against HIV-1, as measured against panels of primary isolates, than do short-term non-progressors or progressors (Pilgrim et al., 1997). It is unknown, however, whether the broader neutralizing antibodies are the cause or an effect of long-term non-progression.

Neutralization escape mutants have been described in several studies, including a longitudinal study of SIVcpzANT in a chimpanzee. During chronic asymptomatic infection, escape mutants emerged an average of 15 months after a neutral-

izing antibody response was detected, and new neutralizing antibodies to the escape mutants were detected an average of 8 months later (Nyambi et al., 1997). There is a similar lag time between neutralization and escape in humans; sera are able to neutralize viral isolates from earlier timepoints, but not the contemporaneous isolate (Arendrup et al., 1992; Lathey et al., 1997). Another study found HIV-1 infected hu-PBL-SCID mice passively administered with high levels of potent neutralizing antibodies developed neutralizing escape mutants within a few days, which correlated with an increase in viral load (Poignard et al., 1999). Neutralization escape mutants do arise in long-term non-progressors (Bradney et al., 1999), indicating that, unlike CTL escape, escape from antibodies is not correlated with progression. The existence of escape mutants indicates that neutralizing antibodies exert selective pressure on HIV-1, although the low titers of antibodies and long time periods for escape mutants to emerge suggest that the selective pressure is likely weak.

4.4. Drug therapy

Combinations of antiretroviral drugs can be very potent in controlling HIV replication. Current antiretroviral therapy most commonly targets two viral enzymes, RT and protease (PR). In response to suboptimal drug therapy, variants are selected that have mutations in the targeted genes which lead to a reduction of drug sensitivity. Individuals in whom these variants arise then fail the drug therapy. Approximately 30–50% of HIV-1 infected individuals outside of clinical trials fail highly active antiretroviral therapy (Fatkenheuer et al., 1997; Deeks et al., 1999). This high rate of failure raises the issue of the spread of drug-resistant viral strains through the human population, which would undermine currently available treatment options.

Transmission of HIV-1 strains resistant to RT inhibitors (Erice et al., 1993; Quigg et al., 1997) or to both RT and PR inhibitors (Hecht et al., 1998) has been documented. Multiple studies have investigated the frequency of resistance-related mutations in HIV-1 from recently infected individuals (Birk and Sonnerborg, 1998; Fontaine et al., 1998; Gomez-Cano et al., 1998; Pellegrin et al., 1999). Drug susceptibility testing has shown that up to 26% of therapy-naïve individuals harbored populations with a modest (3-to 10-fold) reduction in susceptibility to at least one drug (Boden et al., 1999; Little et al., 1999; Yerly et al., 1999), although the clinical significance of this observation is unclear. Because known primary resistance mutations are frequently absent in population-based sequencing of these infected individuals, isolates with such modest resistance likely represent background variability of drug sensitivity (Little et al., 1999). However, between 2 and 16% of the individuals studied did have viral populations with greater than 10-fold reduced susceptibility to one or more drugs, along with primary resistance mutations. These latter data suggest that drug sensitivity testing prior to treatment of drug-naïve individuals may become warranted.

4.5. Compensatory mutations

Initial adaptation of an HIV-1 population to strong selective pressures can result in a compromised replication rate. In subsequent rounds of replication under continued selection, the occurrence of second-site mutations that compensate for part or all of the lost fitness can be observed. This phenomenon has been most clearly demonstrated for the evolution of resistance to protease inhibitors. Some mutations implicated in resistance to protease inhibitors reduce viral fitness (Croteau et al., 1997; Mammano et al., 1998; Martinez-Picado et al., 1999). A possible explanation for the lower fitness associated with lower susceptibility to protease inhibitors is the reduced binding affinity of the protease mutants for the substrate as well as the inhibitor (Schock et al., 1996). Viral genomes with mutant protease genes can regain fitness by two mechanisms. First, mutations outside the protease active site can increase the catalytic efficiency of protease by increasing the turnover number without changing the reduced inhibitor binding (Pazhanisamy et al., 1996; Schock et al., 1996). Second, mutations at one or more protease cleavage sites can occur to enhance the rate of cleavage of the substrate by

the attenuated enzyme, as has been shown for the Gag cleavage sites in nucleocapsid-p1-p6 (Doyon et al., 1996; Zhang et al., 1997b; Mammano et al., 1998). Compensation for reduced activity due to inhibitor resistance has also been demonstrated for RT (Boyer et al., 1998).

It should also be pointed out that the in vitro generation of second-site revertants of mutations introduced into the HIV genome is a powerful genetic approach to map physical or functional interactions between different elements of HIV. For example, this method has been used to map RNA secondary structures (Garcia et al., 1989; Emiliani et al., 1996; Berkhout et al., 1997), and to examine interactions between p17/MA and gp41 (Mammano et al., 1995) and between HIV genomic RNA and Gag (Liang et al., 1999; Paillart and Gottlinger, 1999).

5. Summary

The study of HIV-1 sequence variability will continue to represent an important strategy for understanding the selective pressures on the virus and the mechanisms of escape from those selective pressures. Virus evolution over time and the presence of co-existing viral populations within a person point to dynamic interactions between the host and virus. These interactions include host immune responses, evolution of coreceptor use, and compartmentalization. Initial studies have shown a clear correlation between viral diversity and disease progression, and an understanding of this relationship will become an integral part of our perception of the disease of AIDS.

Escape from selective pressure will always be represented by specific sequence changes. Thus, genetic analysis will continue to be a primary tool for defining the nature of drug resistance and will become increasingly linked to understanding the host immune response. An important underlying question is the extent to which a small effective population size may be fixing mutations through stochastic processes as opposed to a large effective size driven only by selection for fitness.

Finally, the study of sequence variability will assume an important role in vaccine trials. An important component of understanding vaccine efficacy will be correlating sequence divergence within the transmitting population with vaccine failures to define the role of virus heterogeneity in vaccine success/failure. Thus, the study of HIV sequence diversity within and between people will continue to provide us with important insights as we struggle against this pathogen.

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