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Genetic Drift Can Dominate Short-Term Human Immunodeficiency Virus Type 1 *nef* Quasispecies Evolution In Vivo

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The evolution of human immunodeficiency virus (HIV) type 1 *nef* quasispecies in a patient clonally infected with a contaminated batch of blood clotting factor IX was monitored. *nef* sequences were derived at 11, 25, and 41 months postinfection from infected peripheral blood mononuclear cells after molecular cloning of PCR-amplified proviral DNA. The phylogenetic relationships among a total of 41 informative sequences were established by split decomposition analysis and used as a basis to establish a substitution matrix and to score synonymous (s) and nonsynonymous (ns) substitutions. The number of observed in-phase stop codons within the *nef* sequences was comparable to that expected on a random basis. Similarly, the numbers of observed s and ns substitutions did not differ significantly from expected values. No codon position was preferentially mutated. The maximum sequence divergence increased in a linear manner, with ~4.4 nucleotide and ~3.2 amino acid changes per year. It appears that stochastic processes strongly influence short-term HIV *nef* quasispecies evolution in vivo.

Infection with human immunodeficiency virus (HIV) is frequently a clonal event (58, 87, 88). Subsequent error-prone replication leads to the establishment of a complex mixture of genetically diverse but related viral genomes called quasispecies (20, 29). Within their hosts, HIV quasispecies fluctuate in both time and space (17, 49, 67, 74); however, the factors that drive these fluctuations and their relative contributions are not well defined.

A number of forces might contribute to intrapatient HIV evolution. With regard to selection processes, the competitive ability of variants could influence their relative abundance within the quasispecies (12). While negative selection would reduce the frequency of low-fitness mutants, positive selection would ensure efficient outgrowth of mutants with increased fitness (41). Secondly, given the requirement of cellular activation for HIV replication, factors like antigens and cytokines, which stimulate permissive CD4-positive T lymphocytes and macrophages, could influence virus growth (23, 44, 70). Such processes would be governed by chance because there is no biochemical link between the nature of the antigen or cytokine and the expanding HIV variant. Finally, there is bottlenecking, which may reflect stochastic events. Only a few infected cells are expected to successfully support HIV replication, while most are destroyed by the intense polyclonal antiviral immune response (40, 52, 78). This situation is analogous to virus passaging with a low multiplicity of infection in vitro, which spurs genetic diversity (53, 68).

The relative importance of selection events and random processes for intrapatient HIV quasispecies evolution might be estimated from the proportion of synonymous (s) and nonsynonymous (ns) substitutions accumulating during infection. ns substitutions are favored under conditions of continuous positive selection, whereas s changes are expected to be the most frequent if negative, purifying selection dominates. In the case of genetic drift, the proportion of the number of s substitutions per s site to the number of ns substitutions per ns site should be close to 1. With such an analysis the positive selection during the evolution of the peptide binding regions of the major histocompatibility complex genes has been well established (33, 34). However, a similar analysis of intrapatient HIV evolution might cause problems. Due to the high error rates of RNA viruses and retroviruses, there is a high probability that parent and daughter sequences exist simultaneously within an infected individual. Consequently, single mutation events might be counted many times, leading to incorrect s- and ns-substitution ratios. Such problems should be minimized if the scoring of s and ns substitutions is based on the phylogenetic relationship of HIV variants.

A recently developed mathematical clustering technique, split decomposition (4), has proved appropriate for the analysis of RNA virus evolution (18). Split decomposition is a nonapproximative method by which a set of virus sequences in the form of a distance matrix is decomposed into a number of binary splits. The splits can then be presented as a network in which the nodes and tips of the branches correspond to individual sequences (18). By knowing the clonal origin of an HIV infection or by assuming that it is represented by the node with the most branches at an early time point after the primary infection, it should be possible, first, to make a reliable estimation of the number of s and ns substitutions along the evolutionary path and, second, to make an estimation of the number of s and ns substitutions that would be expected in a randomly dominated evolutionary process. Comparison of the observed and expected numbers of s and ns substitutions should then yield an idea about the contributions of the various mechanisms for HIV evolution in vivo.

A number of studies on sequential intrapatient variation of HIV quasispecies have been reported (3, 10, 16, 30, 31, 41, 42, 47–50, 64, 71, 72, 84, 85). Nevertheless, phylogenetic analyses of the evolutionary pathways have been rare and in most cases comprise too small a data set compared to what would be needed for the statistical evaluation of phylogenetically derived s- and ns-substitution ratios. An additional problem is the

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quasispecies diversity in late-stage seropositive patients, where the reconstruction of the evolutionary links between the sequences is often ambiguous due to the extent of evolution and variant turnover.

A study which aimed to analyze factors that would contribute to intrapatient HIV type 1 (HIV-1) *nef* quasispecies evolution by phylogenetically based s and ns substitutions was therefore undertaken. The *nef* gene was analyzed for the following reasons: (i) *nef* is sufficiently variable to allow statistical evaluation of s and ns changes (5, 16, 32, 48, 71), (ii) there is strong selection for an intact *nef* gene in vivo (36), (iii) Nef is highly immunogenic for cell-mediated immunity (13, 14, 25, 38, 43, 65), and (iv) *nef* variants show profound differences in replication rate and virus pathogenicity in vivo (15, 19, 36). Sequential intrapatient *nef* quasispecies from a hemophiliac, patient M, who had previously been shown to be clonally infected with HIV-1 through a contaminated lot of factor IX (10) were analyzed. Although the preparation had been given to at least 48 individuals, only 9 became infected. This low seroconversion rate, together with the intrapatient *env* gene homogeneity in three individuals shortly after primary infection, has been taken as evidence for a clonal infection event (10). By analysis at early time points thereafter, it was possible to unravel the evolutionary pathways. The obtained data emphasize the importance of stochastic events in *nef* quasispecies evolution in vivo.

MATERIALS AND METHODS

Patient material. Patient M is a hemophiliac who had been clonally infected with HIV-1 through a contaminated batch of factor IX in 1989 (10). Up to now he remained clinically well, with CD4 cell counts of 1,204, 922, and 912 per μl at 11, 25, and 41 months postinfection, respectively. Proviral copy numbers were approximately 100 per μg of DNA. The patient received no antiviral treatment.

Amplification of HIV-1 *nef* proviral DNA. The HIV-1 *nef* region was amplified from 1 μg of total DNA of infected peripheral blood mononuclear cells (PBMC) by nested PCR with the outer primers nef1, 5′-GGAGGGTGACCAGTAGCT GAGGGGACAGATAG, and nef2, 5′-CCAGTCGACCAGAGTCACACAAC AGACGGG, and the inner primers nefup5, 5′-GGATCCGAATTCTAAGAC AGGGCTTGGAAAGG, and nefup3, 5′-GTCGACCTGCAGAGTCCCCAGC GGAAAGTCCC. PCR mixtures contained 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 μM (each) deoxynucleoside triphosphate, 0.4 μM (each) primer, and 2.5 U of *Taq* polymerase (Perkin-Elmer) in a total volume of 100 μl. One-tenth of the product from the first round of amplification was used for the second PCR. Cycling parameters were 25 and 30 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C for the first and second rounds, respectively, followed by a final elongation step of 10 min at 72°C. Before amplification, the samples were denatured for 5 min at 94°C. With this amplification protocol, less than 0.3 substitution per *nef* gene are generated by the *Taq* polymerase. To prevent sample contamination, the isolation of HIV-containing DNA, setup of the PCR, and subsequent PCR were all performed in separate laboratories.

Cloning and sequencing. To verify *nef* gene amplification and to estimate product yield, 1/10 of the PCR mixture was run on a 1.5% agarose gel. Approximately 50 ng of *nef* DNA was subsequently ligated with the TA cloning plasmid pCRII (Invitrogen). Competent *Escherichia coli* INVaF′ cells were then transformed and screened for white colonies on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) indicator plates. Isolated plasmid DNA (QIAwell 8 Plus plasmid kit; Qiagen) was digested with *Eco*RI and screened for *nef* gene inserts on 1.5% agarose gels. For each time point, about 20 colonies were sequenced with the *Taq* dye terminator cycle sequencing kit (Applied Biosystems). The products of the reactions were then analyzed on an Applied Biosystems model 373A sequencer. Sequencing primers were standard M13 reverse and M13 universal primers as well as internal *nef*-specific primers (nefS7, 5′-GGACTGGA AGGGCTAAT; nefS8, 5′-GCCAATCAGGGAAGTAG).

Analysis of the sequence data. Multiple-sequence alignments were performed with CLUSTAL V (26), and Hamming distances were calculated with DISTANCES from the HUSAR sequence analysis program package provided by the German Cancer Research Center in Heidelberg.

A parsimony phylogenetic tree was calculated from the *nef* sequences and plotted with the programs SEQBOOT, DNAPARS, CONSENSE, and DRAWTREE of the PHYLIP 3.5c program package (21a). The unrooted tree was determined after 13 randomizations of the sequence input order. One hundred rounds of bootstrapping were performed to assess statistical robustness.

To establish the phylogenetic relationship between sequential *nef* quasispecies, the split decomposition method of Bandelt and Dress was applied (4). Gaps were excluded from the analysis. The aligned *nef* sequences were reformatted with READSEQ and analyzed with the program SplitsTree (34a). The method decomposes the sequence dissimilarity of the data set by bipartitioning (splitting) the set into sequence subfamilies. The obtained splits suggest how individual sequences might be phylogenetically related. The significance of the topology was verified by bootstrap resampling (1,000 replicates). For graphic constructions the Hamming distance matrices were used. Matrices based on Kimura's correction formula (37) did not alter the calculated phylogenetic relationships.

Nucleotide sequence accession numbers. The *nef* sequences described in this study have been deposited in GenBank; the accession numbers are U52465 to U52512.

RESULTS

Temporal increase of intrapatient HIV-1 *nef* gene complexity. The HIV-1 *nef* quasispecies of patient M were established from PBMC obtained at 11, 25, and 41 months postinfection. The aligned protein sequence data set is shown in Fig. 1. At 11 months only 3 of 18 sequences were identical. The other variants displayed mainly single substitutions. The subsequent quasispecies increased in complexity, with a maximal divergence of 5.3% at the protein level. Mutations were scattered throughout *nef*, arguing against mutational hot spots. Of a total of 48 nonidentical *nef* variants, 6 were obviously defective due to in-phase stop codons (clones 11.20, 25.4, 25.20, and 41.13) or −1 frameshifts (clones 25.4, 25.14, and 25.18). Among nucleotide sequences derived from the month 25 sample, 10 of 17 carried a 27-bp in-frame deletion close to the 5′ end of *nef*. It is possible that the corresponding subsequent amino acid residues are still myristylated, as the second residue is still glycine whereas there is a serine at position 5 instead of the more usual position 6.

HIV-1 *nef* gene evolution can be driven by random processes. To examine the overall similarity of the intrapatient *nef* gene sequences, a phylogenetic tree based on maximum parsimony was constructed. The unrooted consensus tree has been tested for statistical robustness by bootstrapping and is shown in Fig. 2. A temporal clustering of the *nef* sequences is observed, demonstrating complete sequential replacement of the quasispecies.

To establish the phylogenetic relatedness between and within the sequential *nef* quasispecies, the split decomposition method was applied (4). It provides a network presentation of the sequence data set, with individual sequences at the nodes and tips of the branches and the lengths of the connecting lines being proportional to the sequence distances. The phylogenetic relatedness within each data set was established first. This was justified, as the temporal sequence clusters did not overlap (Fig. 2), and necessary, as the decomposition of the complete set of the 48 *nef* sequences could resolve only 52% of the underlying Hamming distance matrix (graph not shown). The SplitsTree program was applied separately to each group of aligned *nef* sequences. Unambiguous phylogenetic relationships were obtained after removal of clones 25.7, 25.9, 25.11, 25.18, and 25.19 from the month 25 data set and clones 41.2 and 41.16 from the month 41 data set (Fig. 3). These sequences were responsible for a low matrix resolution, and their interrelation remains ambiguous. Bootstrapping confirmed the significance of each decomposition result. Though some of the bootstrap values seem to indicate low reliability for the splits, no other alternative splits appeared during bootstrapping. Values of less than 85% belong to extremely short splits, i.e., those representing only one or two mutations. Consequently, these positions have a lower probability of being sampled.

All sequences in the first sample were apparently derived from sequence 11.3 by up to four mutations. In order to identify the phylogenetic connections between the different *nef* quasispecies, the combined data sets were analyzed with the SplitsTree program. Clones 11.1 and 25.10 exhibited minimum

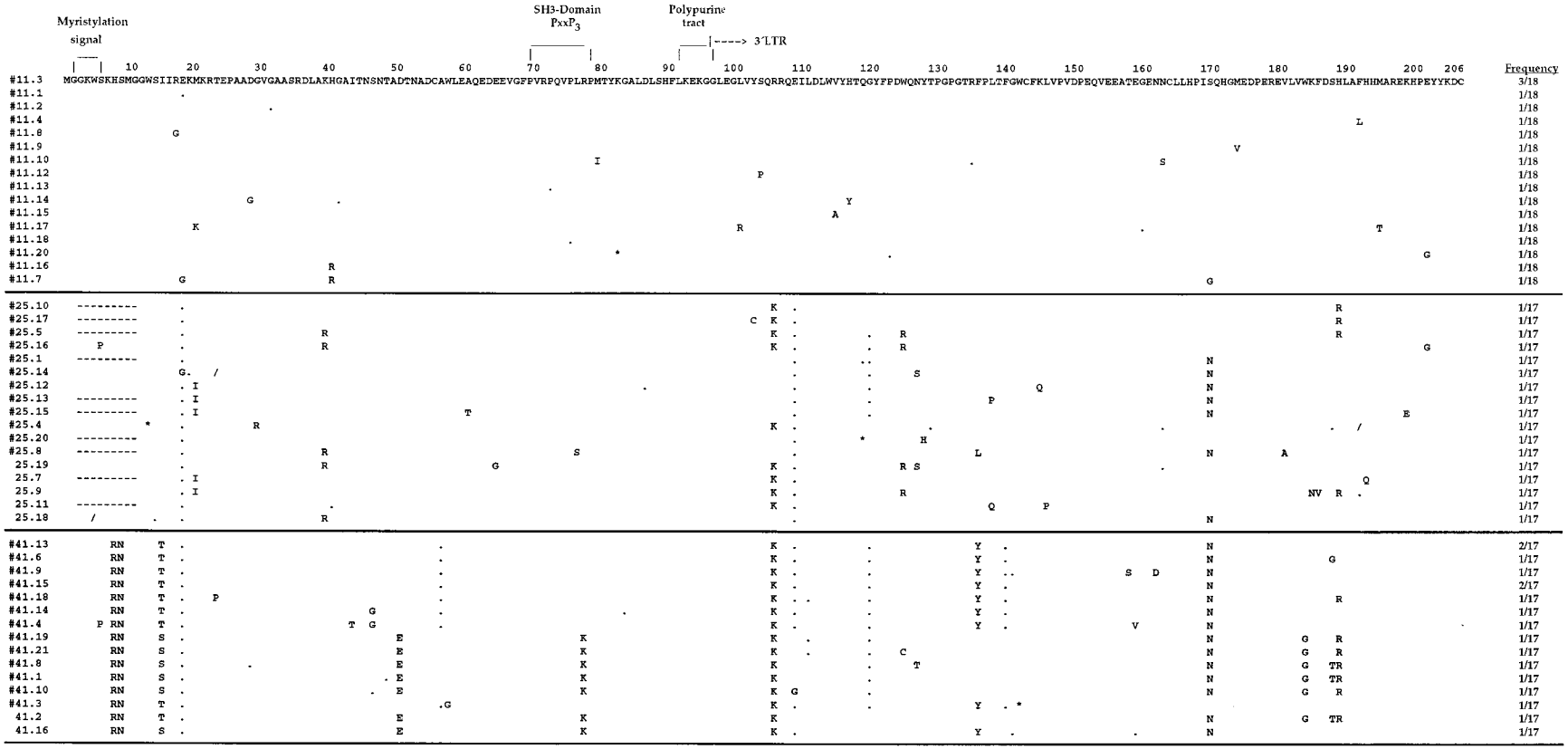

FIG. 1. Nef amino acid sequences corresponding to HIV-1 *nef* quasispecies obtained from patient M 11, 25, and 41 months after clonal infection. Amino acid sequences are aligned with that of clone 11.3 Nef. Clone designations are given on the left, indicating the time point after infection and the clone number. All clones marked with "#" are phylogenetically informative (see also Fig. 3). The right column shows the frequency of the given protein sequence. Only amino acid differences are listed. Dots indicate s substitutions, hyphens represent deletions, slashes indicate −1 frameshifts, and asterisks indicate stop codons. The amino acid one-letter code is as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr, V, Val; W, Trp; and Y, Tyr. LTR, long terminal repeat.

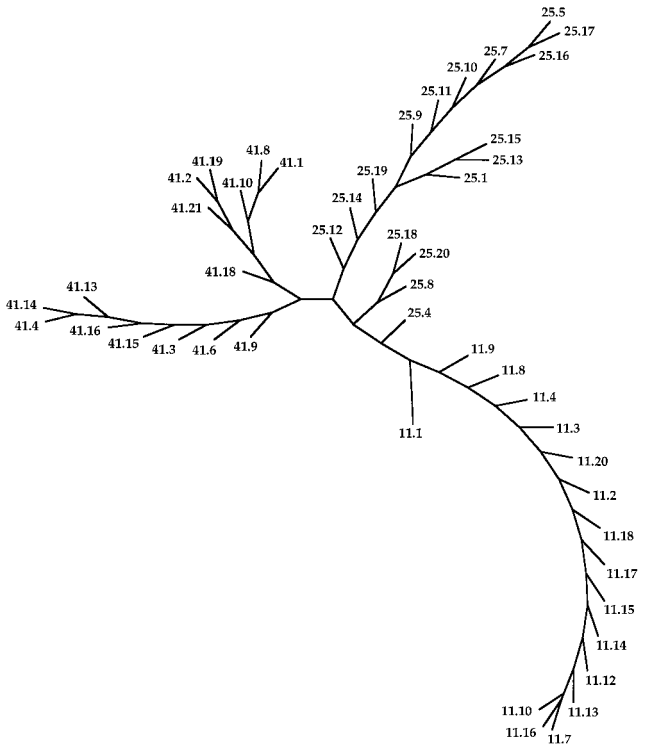

FIG. 2. Maximum-parsimony tree of intrapatient HIV-1 *nef* sequences 11, 25, and 41 months after clonal infection. Statistical significance was confirmed by bootstrapping (100 data sets). The bootstrap values for the two branches separating the month 11 and month 25 clusters and the month 25 and month 41 clusters are 61 and 84%, respectively.

distance between the month 11 and month 25 and between the month 25 and month 41 *nef* sequences, respectively, and were therefore considered to be the respective ancestors of the individual HIV populations (Fig. 3).

Based on the phylogenetic relations of the three data sets, the s and ns substitutions were scored. Excluding gaps from the analysis, a total of 85 substitutions were found, of which 60 were ns and 25 were s changes. The numbers of expected substitutions, 23 s and 62 ns substitutions, were calculated as follows. First, from the phylogenetic-origin *nef* clone, 11.3, the possible s and ns substitutions were scored for all possible transitions and transversions. Second, the numbers of possible transitions and transversions were weighted by the proportion of the observed transitions and transversions, both for s and ns changes. Third, the proportions of these weighted substitution values were then used to calculate the expected numbers of s and ns changes from the total numbers of observed substitutions by multiplication. No significant bias for either s or ns changes could be identified [χ^2 (1 df; 95% confidence interval, 0.05) = 0.25, $P > 0.5$].

The *Taq* polymerase error under the present conditions was lower than 0.3 nucleotide substitution per cloned *nef* sequence (data not shown). Thus, of the total of 85 phylogenetically informative substitutions, less than 12 could be artificial. Assuming that 76% (i.e., a random amount) of these base changes resulted in an ns mutation, nine ns and three s substitutions could be expected. Subtraction of these numbers from the phylogenetically derived total numbers of ns and s substitutions did not alter the significance estimation.

None of the three codon positions showed a statistically significant preference for nucleotide substitutions. Of the 85 phylogenetically informative mutations, 23 were located at the first, 32 at the second, and 30 at the third position. This distribution showed no positional bias ($\chi^2 = 1.6$; $P > 0.5$).

Biased nucleotide substitution during intrapatient *nef* gene evolution. Among the 85 phylogenetically related nucleotide substitutions, a fourfold preference for transitions over transversions was evident, A to G being the most frequent transition (Table 1). The data were normalized to this substitution and to the biased nucleotide content of clone 11.3 (199 A's, 169 G's, 127 C's, and 126 T's). The relative frequencies of A-to-G and T-to-C transitions were comparable and approximately twofold greater than the compensatory G-to-A and C-to-T transitions. On the basis of the nucleotide substitution matrix (Table 1) it was possible to calculate the expected number of in-frame stop codons within the complete set of *nef* gene sequences with clone 11.3 as the origin. Approximately 3.2 stop codons were anticipated, with 4 being observed.

Linear increase of the maximal *nef* gene divergence over time. In the absence of selection, i.e., when the ns- and s-

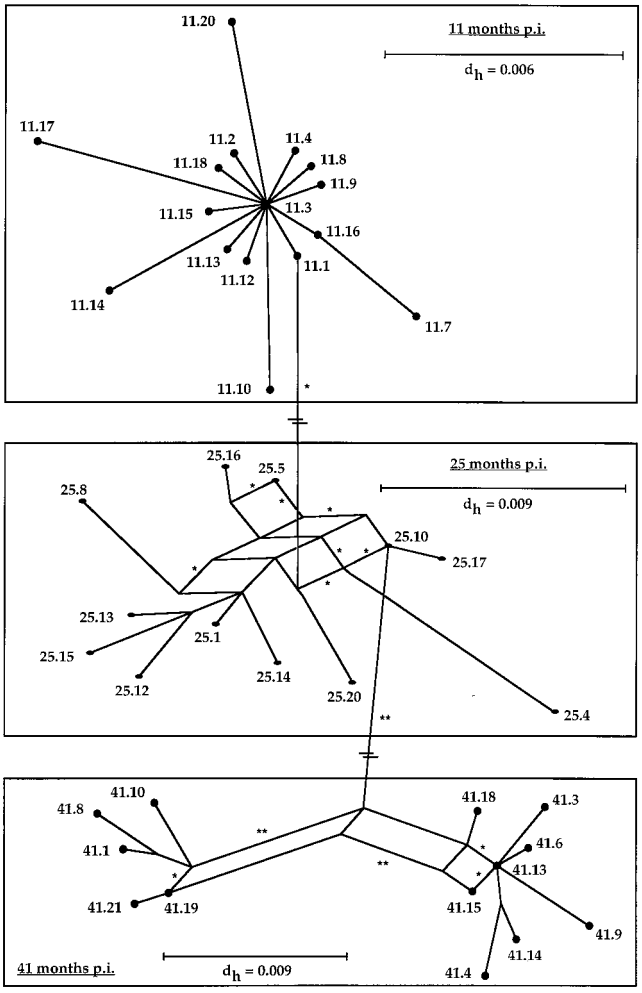

FIG. 3. Phylogenetic analysis of intrapatient *nef* sequences. Sequence relationships were established by the split decomposition method. The time of quasispecies analysis after primary infection is shown. *nef* sequences are represented as dots with the sequence designation. The lines between connected *nef* sequences relate to their respective Hamming distances (d_h). Symbols for statistical-significance levels for interior splits obtained by bootstrapping are as follows: *, 62 to 89% and **, 90 to 100%. The values for the trivial splits are between 61 and 99%. p.i., postinfection.

TABLE 1. Nucleotide substitution matrix[a]

Original nucleotide	Value type	Frequency of change to:			
		A	G	C	T
A	Total		31	3	4
	Normalized		1.0	0.10	0.13
G	Total	13		1	1
	Normalized	0.49		0.04	0.04
C	Total	0	0		7
	Normalized				0.35
T	Total	4	4	17	
	Normalized	0.20	0.20	0.87	

[a] Substitutions were counted from the phylogenetically informative sequences shown in Fig. 3. Values were normalized to the most frequent base substitution (A to G) and to the base composition of the phylogenetic origin, *nef* 11.3 (A, 32.1%; G, 27.2%; C, 20.5%; and T, 20.3%).

substitution ratios are close to the expected values, the fixation rate is equal to the mutation rate. Consequently, the maximum sequence divergence (Hamming distance) is a rough indicator of the maximum number of consecutive rounds of viral replication. Maximum DNA and protein sequence divergences each showed a linear increase as a function of time, tailing back almost to the origin (Fig. 4). This corresponds to 4.4 bases per *nef* gene per year or 7.1×10^{-3} substitutions per nucleotide per year. This value is comparable to nucleotide fixation rates for the HIV-1 and simian immunodeficiency virus (SIV) *env* sequences (1, 3, 8, 10, 35, 57, 61, 84). Given the base substitution rate of the HIV-1 reverse transcriptase of 2.5×10^{-5} per replication cycle (45), and assuming that the fixation rate equals the substitution rate for this locus, a minimum of 280 consecutive replication rounds per year are necessary to obtain a nucleotide divergence of 2.4% at 41 months postinfection.

DISCUSSION

The evolution of the *nef* gene in a clonally infected hemophiliac was dominated by genetic drift. This interpretation is based on the following observations. First, the observed and expected numbers of s and ns substitutions during *nef* quasispecies evolution were not significantly different. Second, in a total of 41 phylogenetically related *nef* sequences, four in-phase stop codons were identified. Taking into account the observed nucleotide substitution bias (Table 1) and the codon usage of the *nef* origin, clone 11.3, this amount is of the same order as the 3.2 stop codons expected on a random basis. Third, no codon position is preferentially mutated. Finally, the observed temporal increase in the maximal *nef* quasispecies divergence was linear. Such behavior is expected to appear only under conditions of continuous error-prone virus replication with little or no positive selection. However, whether the rate of evolution represents a property of the infecting HIV strain or an intrinsic property of the infected individual remains unclear. The recently observed differences in the evolutionary rates of SIVmac239 in four clonally infected macaques suggest the latter (61).

The processes underlying this apparent randomness in HIV-1 *nef* evolution might be related to two properties of HIV, the requirement of cellular activation for growth and the predominance of memory-T-cell infection (23, 69, 70). Expansion of certain variants could therefore depend on the nature of the activating antigen or cytokine, and hence be governed by chance, because there is no biochemical link between the nature of the antigen and the outgrowing HIV variant. Indeed, HIV growth is markedly increased by recall antigens during the contact of virus-pulsed antigen-presenting dendritic cells and CD4 T lymphocytes in vitro (75, 83) as well as by vaccination-induced T-cell activation in seropositive patients (27, 56, 73).

The conclusion that stochastic events determine to a large extent the evolution of lentiviruses in vivo has also been drawn recently from a study of the hypervariable regions 1 and 2 of the SIV envelope gene in clonally infected macaques (61). Four animals had been infected with the molecular clone SIVmac239, and sequence changes were analyzed for up to 19 months postinoculation by a phylogenetic analysis employing Prim's algorithm. Because no preference in the number of ns changes was detected, positive Darwinian selection was assumed not to play a major role in virus variation. Reanalysis of the same data set as well as analysis of the evolution of the V3 region in an HIV-1-infected hemophiliac (30) by the split decomposition approach described here again showed no bias in the number of ns substitutions (data not shown). Thus, the conclusion that random processes present an important factor in the evolution of HIV and SIV is independent of the phylogenetic analysis method used. Furthermore, this phenomenon does not seem to be restricted either to a particular host, macaque or human, or to a particular part of the lentivirus genome, *nef* or *env*. In addition, as the published V3 *env* sequences had been derived from virus RNA in plasma, the results hold true independently of the source of virus material.

Subtle differences in relative fitness have an important influence on the outgrowth of virus variants in vitro (12). Why should this not hold true and lead to positive selection in a natural HIV infection, where the antiviral immune response seems so intense? Indeed, positive selection of HIV variants by the immune response and subsequent escape from recognition have been suggested to play a role in HIV persistence and to be necessary for pathogenesis (6, 54, 55). In terms of the humoral immune response to HIV, however, neutralizing titers of antibodies against primary isolates are much smaller than has been anticipated from earlier work with laboratory-adapted virus strains. The neutralizing capacity is orders of magnitude lower, as it is after, e.g., influenza virus infection

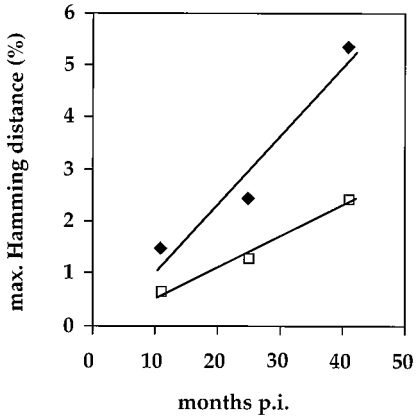

FIG. 4. Sequential increase of the maximal (max.) intrapatient *nef* sequence divergence. The nucleotide (□) and protein (◆) sequence divergence within the *nef* quasispecies is given as the relative Hamming distance at 11, 25, and 41 months after clonal HIV-1 infection. The relative Hamming distance is defined as the number of point mutations between two sequences per sequence length. Correlation coefficients are 0.99 for DNA and 0.94 for protein. p.i., postinfection.

(51), and therefore it is not obvious how it might influence the fitness of particular HIV variants in vivo.

By contrast, the cellular immune response to HIV seems particularly strong and is believed to be a major factor in the decline of viremia early after the primary infection (7, 39, 86). Antiviral cytotoxic T lymphocytes (CTL) even seem to have access to HIV-infected cells, as cooccurrence of HIV replication and expansion of specific antiviral CTL has recently been demonstrated within single splenic white pulps (9). Together with the lymph nodes, these are the centers of antigen presentation and T-cell activation and therefore present the physiological conditions that most favor HIV growth and immune-mediated inhibition in vivo (9, 21, 59). However, the anti-HIV CTL response is usually oligoclonal; more than 15 HIV-specific CTL clones have been identified within a single splenic white pulp (9, 9a). With such a level of oligoclonality of the effector cells and the known promiscuous specificity of epitope recognition (76), the outgrowth of mutants is expected to be severely restricted, as has been recently shown for lymphocytic choriomeningitis virus infection of mice vaccinated against only two dominant CTL epitopes (82).

Besides the direct lysis of HIV-infected cells, CTL can inhibit virus growth through the action of various soluble suppressor factors (2, 11, 80). As recently shown in a transgenic hepatitis B virus mouse model, such an effector mechanism can contribute significantly to virus elimination (24). While the extent to which such factors inhibit HIV in vivo is unknown, their variant, independent, broad anti-HIV activity is expected to further reduce the likelihood of an HIV immune escape.

With a nucleotide fixation rate of 4.4 nucleotides per *nef* gene per year, a 7.1% divergence within the *nef* quasispecies might be found after 10 years of an HIV infection. Such a high level of divergence is in good agreement with the observed intrapatient variability and virus turnover (3, 16, 28, 30, 60, 62, 72, 81, 84). Furthermore, the coexistence of HIV mutants or different virus strains (66, 89) underscores the point that observed variation affects little the fitness of a particular virus variant in vivo. This hypothesis is in agreement with recent observations in the SIV macaque model. A single amino acid change in the *nef* region significantly altered the replication properties of SIVmac239 in vitro and led to an acute lethal disease in macaques upon infection (19). The quasispecies nature of the SIV infection predicts that such a highly pathogenic variant should be present in vivo shortly after an SIVmac239 infection; however, no evidence for its outgrowth has ever been obtained.

The nucleotide substitutions were asymmetric, with a twofold excess of A-to-G and T-to-C substitutions with respect to the compensatory transitions, reinforcing a similar observation for the SIVmac239 V1/2 region in the *env* gene in macaques (61). As lentivirus genomes have an uneven nucleotide composition (typically 33 to 39% A's, 17 to 22% C's, 18 to 26% G's, and 20 to 26% T's), this mutational bias must be somehow compensated for. G→A hypermutation of the lentiviral genome, a consequence of reverse transcription in the presence of biased deoxynucleoside triphosphate concentrations, is a possibility, as it may occur on both strands, has the opposing transition preference (22, 35, 63, 77, 79), and is not incompatible with gene function (46).

Although analysis of the nucleotide substitutions in phylogenetically based HIV and SIV sequences in vivo revealed substitution biases, there was no evidence of strong selection. This held for different genomic regions (*env* and *nef*), different sources of the viral sequences (infected lymphocytes and cell-free virus), and different species (humans and monkeys). Thus, the importance of immunological selection events for virus variation and the impact of virus variation per se on pathogenesis appear to be minimal. As AIDS almost invariably follows HIV infection regardless of the variant, the key condition for persistence in vivo and presumably for disease appears to be replication competence.

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