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Quasispecies and naturally occurring superinfection in feline immunodeficiency virus infection

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Summary. Analysis of individual clones containing the V1 and V2 domains of the segment of the FIV *env* gene present in a naturally infected cat (T) was carried out. The polymerase chain reaction (PCR) was used to amplify proviral FIV DNA extracted from peripheral blood mononuclear cells (PBMCs) obtained in October 1994 from this cat. The PCR products were cloned and the DNA sequences determined for 11 clones. Sequences obtained were aligned with sequences corresponding to FIV isolates (T90, T91, T92) previously obtained from the same cat in 1990, 1991 and 1992. Phylogenetic analysis was performed which included consensus sequences of another Australian isolate, N91, as well as UK, US, Swiss and Japanese isolates of FIV.

All clones varied from each other, and none of these clones was identical to the consensus sequences of the isolates obtained previously from the same cat (the T-series). However, most of these clones appeared to have originated from the ancestor of the most recent isolate (T92). In addition, 2 of the clones (7&11) are closely related to another Australian isolate N91, obtained from a different cat (N) in 1991. Because these two cats (T and N) were housed together for at least 3 years (1990–1993) it is suggested that the first cat (T) has become superinfected with an isolate from a second cat (N) under natural conditions.

The identification of clones of differing sequences, which were not identical to each other nor to their ancestors, emphasises the rapid mutation of lentiviruses within the *env* region, and the difficulty of developing an effective FIV vaccine. More importantly, the possibility of natural superinfection with FIV in cats has implications for the development of a successful lentiviral vaccine.

Introduction

Nucleotide sequence variation between isolates of human immunodeficiency virus type 1 (HIV-1), particularly in the envelope (*env*) gene, has been widely

studied for its relevance to vaccine development, viral evolution, and pathogenesis [2, 11, 23, 28, 33]. The HIV-1 genome exhibits extensive sequence variation, both between patients and within individual patients at any one point in time [1, 8, 15]. The variation of HIV-1 genomes within an individual is less than that between individuals, with the greatest variation in the surface glycoprotein encoded by the *env* gene [1, 3, 5]. The collection of distinct but related virus variants within an individual has been termed 'quasispecies' [8, 17].

Feline immunodeficiency virus (FIV), a lentivirus related to HIV, has been classified into three subtypes, A, B and C on the basis of phylogenetic analysis of the *env* region [30]. Subtype A includes isolates from California and Europe, subtype B, Japanese and the central and eastern United States isolates, whereas subtype C consists of isolates from south-western Canada. All Australian isolates cluster together within FIV subgroup A with a 95–97% nucleotide homology with the Californian isolates [9].

We have previously described [10], using consensus sequence analysis, the in vivo evolution of FIV isolates of T-origin which were obtained sequentially from a persistently infected cat, "T", over a 3-year period. Here we describe in cat T, the existence of multiple variants (quasispecies) of FIV, which have arisen over the last 5 years. Their phylogenetic relationship to other FIV isolates was also determined.

While the extended clinical latency of lentiviral infections, combined with a continued exposure to infection, raises the question of an individual person or animal becoming infected with further heterologous isolates, there is strong evidence to suggest, at least in HIV infection, that persons infected with one variant are refractory to infection with a second variant [11, 23]. There are however, instances of infection with multiple strains of HIV during the period of initial infection [4, 34]. Furthermore, indirect evidence from the phylogenetic analysis of HIV-1 and HIV-2 subtypes indicates that co-infection of one individual with highly divergent strains occurs [27]. By restriction enzyme analysis, superinfection of a chimpanzee with a second distinct strain of HIV has been demonstrated [6] and we [16], as well as Okada and colleagues [21], have shown the experimental induction of in vivo superinfection in FIV infection. There are no reports of natural superinfection of cats with different strains of FIV.

This current study reports the presence of FIV clones in cat "T" which appeared to have originated from another cat "N". Because cat "N" was housed for a 3-year period with "T", we conclude that "T" has become naturally superinfected with "N"'s virus.

Materials and methods

Virus isolates

FIV isolates T90, T91, and T92 were obtained at yearly intervals from a cat, "T", which has naturally acquired infection. Isolate N91 was obtained from another naturally infected cat "N". Both cats were from Perth, Western Australia and both were housed in common for

varying times over a 3-year period. Consensus sequences of a segment of *env* genes of isolates T90, T91, T92 [10] and N91 [9] are known. Sequences from other FIV isolates used in this study were obtained from published sequences and their GenBank accession numbers, which are shown are in parenthesis, are as follows: U.S. Petaluma isolate (M25381) [22, 32], FIV PPR (M36968) also from the U.S.A. [25], FIV TM2 (M59418) from Japan [19], FIV Z1 (X57002) from Switzerland [20], and FIV UK 8 (X69496) from the U.K. [26].

Preparation of cellular DNA

Peripheral blood mononuclear cells (PBMCs) obtained from cat "T" in October 1994 were purified by Ficoll-Hypaque density gradient centrifugation of heparinised whole blood. PBMCs were washed twice in phosphate buffered saline (PBS), and total genomic DNA extracted as described previously [16]. Briefly, PBMCs (approx. 1×10^6) were lysed in nucleic acid lysis buffer (10 mM Tris-Cl, pH 8; 2 mM EDTA, pH 8; 400 mM NaCl; 5% SDS); and digested with 100 µg/ml proteinase K (Boehringer Mannheim, Federal Republic of Germany). The cells were incubated at 55 °C for several hours and the proteinase K subsequently inactivated at 95 °C for 10 min. Proteins were then precipitated with 100 µl of saturated NaCl [18]. DNA containing supernatant was precipitated in ethanol, and the pellet obtained washed with 70% ethanol, and resuspended in 20 µl of sterile distilled water.

PCR

PCR primer pairs which encompassed the region between L6299 and R6866 were used to amplify a fragment of 568 bases. L6299 is located at positions 6299-6319 of *env* (gp120) and has the sequence 5′ AGGACCAGAAGAAGCTGAAGA 3′, while R6866 is located at positions 6866-6846 of *env* (gp120) and has the sequence 5′ TTCTGGTGCCCAACA-ATCCCA 3′. Amplification of FIV proviral DNA sequences was achieved using the PCR as marketed by Perkin Elmer Cetus (Norwalk, CT). Purified double-stranded genomic DNA (approx. 1 µg) was added to 25 µl of total reaction mix. The reaction mix contained a final concentration of 10 pmole of each primer, 200µM of each dNTP, and reaction buffer (50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM $MgCl_2$, and 0.1 mg/ml gelatin). The reaction was overlayed with paraffin oil, and cycled on a Corbett thermocycler (Corbett Research, Australia). After heat denaturation at 94 °C for 5 min, 1 unit Taq polymerase (Perkin Elmer Cetus, USA) was added and the primers annealed at 60 °C for 1 min, extension was carried out at 72 °C for 2 min and denaturation at 94 °C for 30 sec. At the end of the 30th cycle, extension was carried out at 72 °C for 8 min. PCR products were resolved on 4% NuSieve agarose (FMC BioProducts, USA) gels stained with 0.5 µg/ml ethidium bromide.

Cloning of PCR-products

Bands corresponding to 568 basepairs were excised and gel purified with β-agarase (New England BioLabs, USA) and cloned into pGEM-T vector (Promega, USA). Following transformation into *Escherichia coli* (JM109 cells; Promega, USA), recombinants were selected on plates containing IPTG and X-gal (Boehringer Mannheim). The identity of positive clones were confirmed by restriction endonuclease digestion of plasmid miniscreen DNA.

DNA sequencing

Sequencing reactions were performed by the dideoxynucleotide chain termination method using a M13 universal sequencing primer and taq Dye Deoxy Terminator cycle sequencing

kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The sequence was resolved on an Applied Biosystems model 373A automated DNA sequencer. Sequences obtained were screened and aligned using the Australian National Genomic Information Service (ANGIS).

Phylogenetic analysis

Phylogenetic analysis of nucleotides by Clustalw [12] and neighbour-joining methods [29] were performed on the segment of env region (516 bp), and a tree for all clones obtained from this study as well as the consensus sequences of Swiss (Z1), American (Petaluma and PPR), UK (UK8) and Australian (T90, T91, T92 and N91) isolates were constructed using Japanese strain (TM2) as an outgroup sequence.

Rates of evolution

Rates of evolution, expressed as nucleotide substitutions per site per year, were calculated using the method of Gojobori and Yokoyama [7] where R = D/2T and R = subs/site/year, D = $-3/4$ ln (1–4/3P), T = time since divergence, and P = the proportion of different nucleotides.

Results

Sequence variation

In previous studies [10, 16], we analysed a 5′ segment of the FIV *env* gene coding for an N-terminal region of surface region (SU) spanning the first and second variable domains. In the present study, we analysed the same region to allow direct comparison with our previous data.

DNA containing FIV provirus was obtained from PBMCs of a naturally infected cat, "T" in October 1994, subjected to PCR amplification, molecularly cloned and eleven individual clones isolated and subjected to nucleotide sequence analysis. T90, T91 and T92 are consensus sequences from virus isolates obtained at yearly intervals from the same cat "T" [10]. Results obtained from the current study show that there were a total of 227 nucleotide changes,distributed amongst 11 clones studied. None of the clones was identical to those of T90, T91 or T92. The changes corresponded to a mutation frequency of 4.11×10^{-3}/substitution/site/year. However, none of the clones showed any deletions or insertions when compared with each other.

Translation of the nucleotide sequences to their respective amino acid sequence showed that 136/227 of the nucleotide changes led to amino acid changes. Nucleotide sequences of 9 of the 11 clones isolated revealed that although they were similar to each other as well as to T90, T91 and T92, none of them was identical, thus resulting in heterogeneous protein population. The multiple alignment of the amino acid sequences is shown in Fig. 1.

Phylogenetic groupings

Phylogenetic analysis of the nucleotide sequences by Clustalw [12] and neighbour-joining methods [29] was performed, and a tree for all clones obtained

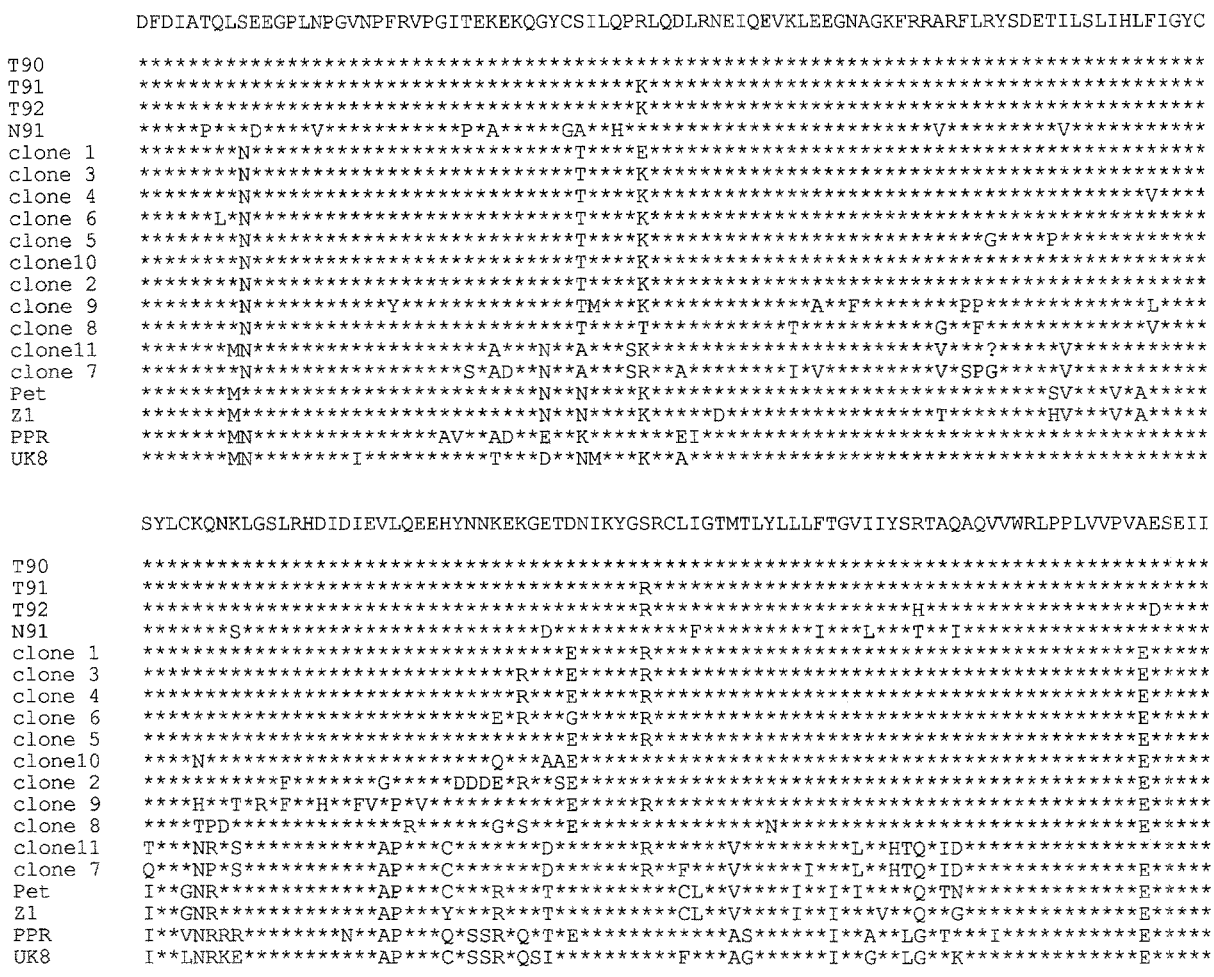

Fig. 1. Amino acid sequence alignment. Comparison of deduced amino acid sequences of an N-terminal region of *env* gene segment from isolated clones with those of Australian isolates (T90, T91, T92, and N91) and other FIV isolates (Pet, PPR, Z1, and UK8). Asterisks indicate sequence identity with T90

from this study as well as the consensus sequences of Swiss (Z1), American (Petaluma and PPR), UK (UK8) and Australian (T90, T91, T92 and N91) isolates were constructed using Japanese strain (TM2) as an outgroup sequence (Fig. 2). This analysis shows that the tree can be separated into two major groups; I and II. Group I contains only the Japanese isolate, TM2, which is now viewed as the FIV prototype B [30]. Other nucleotide sequences included in this phylogenetic analysis may be placed together into another group (prototype A). However, within this second group, a number of clusters are observed, namely UK8 and PPR, Z1 and Pet, clustering as one subgroup.

The pairwise percentage similarities of all clones as well as those of the consensus sequences are shown in Table 1. All clones from T, with the exception of clones 7 and 11, clustered together, differing in nucleotide sequence by

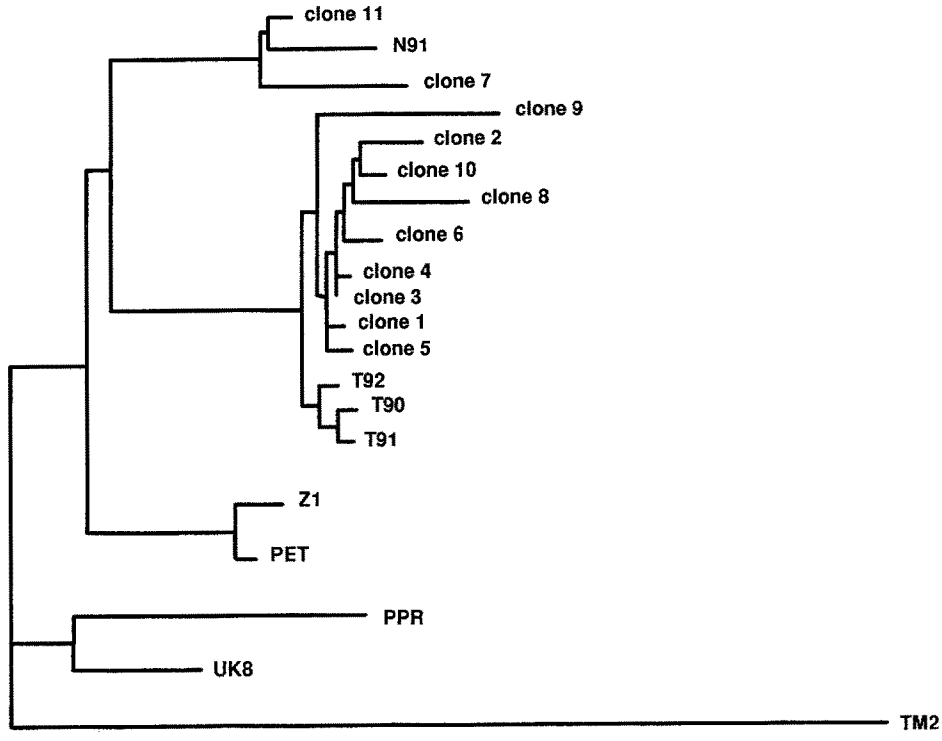

Fig. 2. Phylogenetic tree of FIV based on the nucleotide sequence of *env*. The relationship between the 516 bp of FIV *env* was examined using Clustalw [12] and neighbour-joining methods [29]

a maximum of 7% from each other. The configuration of the phylogenetic tree illustrates that all clones, except 7 and 11, evolved closely with, or possibly from, T92. Within this group, further relationships are evident, for example, clones 2 and 10 are more closely related to each other with an homology of 97.1% than to other sequences (Table 1). Clone 8 has a common ancestor with clones 2 and 10, and has homologies of 94.8% and 95.7%, respectively and clone 6 may have derived from the ancestor of clones 2, 10 and 8. Clones 3 and 4 (99.6% homology) are clustered as one subset while clones 1 and 5 are as another subgroup (99.0% homology). Clone 9 shows a common ancestor with clone 2 through clone 5. Overall, all clones, with the exception of 7 and 11 appeared to have derived directly from the ancestor of original T isolates (T90, T91 and T92).

An unexpected feature of the current study was that clones 7 and 11 differed by 12.8% and 10.3%, respectively, from the most recent FIV isolate (T92) from cat "T". These clones are most closely related to another Australian isolate, N91, obtained from cat "N". The sequence homology of clones 7 and 11 with N91 is 93.2% and 96.3% respectively, indicating that cat "T" has become infected with a second strain, N91. Although T-derived isolates (T90, T91 and T92) are in the

Table 1. Percent nucleotide similarity between *env* regions (6299–6866) of FIV isolates

1: TM2																			
2: UK8	75.0																		
3: PPR	74.5	89.0																	
4: clone 11	75.4	88.2	84.7																
5: N91	73.5	86.4	83.3	96.3															
6: clone 7	72.5	85.3	82.2	95.3	93.2														
7: PET	75.4	88.8	84.7	90.5	88.8	87.6													
8: Z1	75.4	87.8	83.5	89.3	87.6	86.4	98.1												
9: T90	72.9	86.8	82.8	88.6	88.4	86.4	88.4	88.0											
10: T91	73.5	87.4	82.9	89.1	88.6	86.6	89.0	88.6	89.4										
11: T92	73.9	87.6	83.1	89.7	88.4	87.2	89.5	89.1	98.6	99.2									
12: clone 9	72.3	84.3	79.7	85.5	83.3	84.5	84.9	84.5	93.2	93.8	93.8								
13: clone 1	74.3	88.2	83.7	89.3	87.2	86.8	89.0	88.6	97.9	98.4	98.4	94.6							
14: clone 5	74.1	87.6	83.7	89.1	87.0	87.2	88.8	88.8	97.7	98.3	98.3	94.4	99.0						
15: clone 3	74.5	88.0	83.7	89.5	87.4	87.0	89.1	88.8	98.1	98.6	98.6	94.8	99.4	99.2					
16: clone 4	74.7	87.6	83.3	89.1	87.0	86.6	88.8	88.4	97.7	98.3	98.3	94.6	99.0	98.8	99.6				
17: clone 6	74.1	87.0	82.8	88.6	86.4	86.0	88.2	87.8	97.1	97.7	97.7	93.8	98.4	98.3	99.0	98.6			
18: clone 8	71.9	85.3	81.4	86.6	84.7	85.7	86.4	86.0	95.0	95.0	95.0	91.7	95.7	95.9	96.3	96.3	95.7		
19: clone 10	74.1	87.6	83.1	89.0	86.8	86.4	88.6	88.2	97.3	97.5	97.5	94.0	98.6	98.1	98.4	98.1	97.7	95.7	
20: clone 2	72.7	85.9	81.8	87.6	85.5	85.3	87.0	86.6	96.1	96.3	96.3	93.0	97.1	96.9	97.7	97.3	97.1	94.8	97.1

same FIV type grouping as N91, they differ in their nucleotide sequences within *env* gene by almost 12%. Phylogenetic analysis showed that clone 11, with an homology of 96.3% with N91, may have originated from N91 isolate earlier than clone 7.

All Australian isolates (T-series and N91) as well as all clones obtained from this study share a common ancestor with Pet and Z1 isolates, all of which in turn show some similarity to PPR and UK8. As it is well documented, TM2, although still sharing a common ancestor, is most distantly related to all other FIV isolates.

Discussion

In the present study, we determined the nucleotide sequences of a V1–V2 segment of *env* region for FIV provirus clones derived from PBMCs obtained from a cat which had been naturally infected for at least 5 years. This region of the FIV genome was chosen because of its variability as we have previously shown [10]. Sequences obtained from each of the individual clones were then analysed to determine: (1) if a number of proviral strains were present in the one cat, (2) if any of these clones originated from the most recent isolate, T92, and (3) if there was any evidence of earlier isolates (T90 and T91), which were isolated in 1990 and 1991, respectively.

The variation amongst sequential isolates by consensus sequencing obtained from cat "T" [10] over a 3-year period (1990–1992) showed that the base changes in isolates T90–T92 were directional with no nucleotide reversion to that of the original isolate. All changes, once acquired, were retained and this was also evident at the amino acid level. In our present study, individual clones from the same cat have been analysed. It may well be that if clonal sequence analysis was performed with our previous studies, quasispecies, some of which retained T90 or T91 identities, as well as those of T92 sequence, may have been demonstrable at the time of our previous yearly studies. It is possible that each of T90, T91 and T92 isolates were the predominant strains within cat "T" at the time and the most easily recoverable by cocultivation. Without molecular cloning, the existence of minor quasispecies would not have been detected.

The results from our current studies unequivocally show that the majority of clones recovered are members of a cluster which is of T-origin. Each clonal sequence, with the exception of clones 7 and 11, was similar, but not identical to each other or to the T-series. This demonstrates the emergence of quasispecies, the diversity of the virus and the possible deletion from the host of the earlier strains of the virus (T90, T91 and T92).

Two of the clones examined, 7 and 11, resembled most closely another Australian isolate, N91. Indeed, the phylogenetic analysis confirmed that they both originated from N91, which was obtained from another naturally infected cat, "N". Although T-isolates (T90, T91 and T92) and N91 belong to the same FIV prototype, they are clearly distinguishable, particularly in the *env* region of the genome and are considered to be entirely different isolates [10]. It was rather

surprising to find that clones 7 and 11 have the N series as their progenitor, rather than being direct derivatives of T-origin isolates. However, the fact that these two cats, "T" and "N" were housed together for varying times over 3 years may explain this phenomenon of natural superinfection.

We have previously demonstrated the induction of experimentally-induced in vivo superinfection [16], using large doses of challenge virus, as has Okada's group [21]. Thus far, the phenomenon of in vivo superinfection under natural conditions in HIV, SIV and FIV infection is either rare [4, 34] or unreported although the existence of closely related, but non-identical isolates, known as quasispecies, within an individual has been well documented [8, 15, 23].

The development of an HIV vaccine presents a major challenge because of the high mutation rate and the variability in the *env* region of the gene. Our present study reinforces this concept for another lentivirus where a high level of variation exists in the *env* region of the genome within a cat persistently infected with FIV for at least 5 years. Although closely related, none of the individual clones examined was identical to each other nor were they to their ancestors.

Given this *env* region variation, which is in many senses expected, to our knowledge, no study has shown the possibility of an infected cat becoming superinfected with a second more distantly related FIV isolate under natural conditions. That 2 of the 11 clones examined have originated from the superinfecting FIV isolate must now be taken into consideration in the development of a successful lentiviral vaccine when FIV is used as the animal model. It is apparent from this as well as our previous study [16] that cats infected with FIV are unable to resist infection with a second strain. This has important implications with respect to protective vaccines. Furthermore, there is the possibility that the superinfecting strain reactivates latently infecting pre-existing provirus as has been shown for HIV-2 and SIV, thus accelerating the progression of the disease [24].

These results also reinforce the possibility of recombination between two isolates in vivo. Recombination is common in retrovirus infections, but only occasionally reported for lentiviruses [14, 16]. Recombination occurs with a frequency of 40% per cycle during reverse transcription in HIV [13] and we have previously shown that recombination occurs in vivo in FIV infection when two isolates are inoculated simultaneously [16]. Finally, the possibility of superinfection under natural conditions, as observed in our present study, suggests that a high dose of virus, as previously used under experimental conditions [16], may not be required for an individual to become infected with a second strain. If this scenario proves to be true, then the pathogenesis of lentiviral infections needs to be re-evaluated.

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