Genetic Diversity of Feline Immunodeficiency Virus: Dual Infection, Recombination, and Distinct Evolutionary Rates among Envelope Sequence Clades

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For the rapid genetic analysis of feline immunodeficiency virus (FIV), we developed a heteroduplex mobility assay (HMA) that utilizes a PCR-amplified fragment of the FIV envelope gene spanning the third and fourth variable regions of the envelope surface protein coding sequence. Viral sequences were successfully amplified from blood specimens from 98 naturally infected cats from Australia, Canada, Germany, Italy, South Africa, and the United States. Eighty were clearly assignable to the A or B envelope sequence subtypes. Three belonged to subtype C, one was dually infected with viruses harboring the A and B *env* subtypes, and several were categorized as outliers to any of the established subtypes or as probable intersubtype recombinants. Some geographic clustering was evident, with subtypes A and B found in greater frequency in the western and eastern regions of the United States, respectively. Subtypes A, B, and C were found on more than one continent, and countries with more than two samples analyzed contained at least two subtypes. The broadest representation of subtypes was found in Munich, Germany, where three subtypes and one virus that was not classifiable by HMA were found. Thirteen samples were selected for DNA sequence determination over the same region of *env* used for HMA. Analysis of all available FIV *env* sequences from this and previous studies revealed the existence of recombinant viruses generated from subtype A/B, B/D, and A/C envelope gene sequences. Subtype A *env* sequences were less diverse than subtype B sequences, although both groups had well-supported clusters. Furthermore, the mutational pattern giving rise to diversification in the two subtypes differed, with the subtype A viruses showing half as many synonymous site mutations compared to subtype B yet showing similar levels of nonsynonymous site changes. These results are consistent with the hypothesis that FIV-B is an older virus group and is possibly more host adapted than FIV-A.

Feline immunodeficiency virus (FIV) was first described as an infection of domestic cats in 1987 (38) and has since also been found in several feral feline species (3, 28, 36). Estimates of the prevalence of FIV infection among asymptomatic domestic cats vary between countries, ranging from 6% in the United Kingdom (21) to 8% in Germany (16), 12% in Japan (22), and 1 to 14% in the United States, depending on age, gender, and risk of exposure (38, 63). Prevalence in sick animals has been found to be two to three times higher (21, 22).

Like other lentiviruses, FIV infects primarily T lymphocytes, macrophages, and other cells of the hematolymphoreticular system (5, 39, 40). Unlike its human and simian counterparts, HIV and SIV, FIV does not use CD4 as its receptor (2, 4, 33). However like HIV, some lab-adapted isolates of FIV appear to use the CXC chemokine receptor CXCR4 (62). FIV infection is associated with symptoms of immunodeficiency, such as weight loss, chronic opportunistic infections, and, less often, neurological abnormalities (11, 63). Because of its parallels to HIV infection, FIV infection of domestic cats has been inves-

tigated as a small-animal model of HIV infection to aid in the study of AIDS pathogenesis, therapy, and vaccine development.

Of considerable interest in the development of HIV vaccines is the problem of rapidly increasing genetic diversity of the virus. The main group of HIV type 1 (HIV-1) strains has been found to be comprised of at least eight envelope sequence subtypes (32), with genetic variation in this gene growing at a rate of about 1% per year (53). Preliminary data consistent with the hypothesis that different HIV *env* subtypes exhibit distinct pathogenic properties have also been put forward (9). A further complication potentially confounding the development of effective vaccines is that cross-clade recombinant viruses are now being detected at an appreciable frequency (43–45, 47).

As a first step towards modeling cross-clade vaccine efficacy and investigating the pathogenic diversity of FIV strains, we have conducted a molecular epidemiological screen to identify and isolate FIV envelope sequence subtypes. We sampled and evaluated blood samples collected in six countries from 98 cats infected with FIV, using a heteroduplex mobility assay (HMA) (8). This assay has previously been used to reconstruct HIV phylogenies, analyze HIV genome variation, and trace epidemiological connections between HIV-infected individuals (1, 7, 8).

PCR-amplified FIV envelope gene fragments were heated and reannealed with analogous products from two reference sequences of each of the A, B, and C *env* subtypes (56) and subjected to polyacrylamide gel electrophoresis. Subtype spec-

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TABLE 1. Sample descriptions and subtype assignments by HMA^a

Sample code	Country	State/province	City	Source	Clinical symptoms	Subtype ^b
USWAabk2	USA	Wash.	Seattle	J. Abkovitz		? (1) ^c
DEBAmick	Germany	Bavaria	Munich	K. Hartmann	Conjunctivitis, stomatitis	? (4) ^c
AUQLbria	Australia	Queensland	Brisbane	W. Robinson		A
AUQLpicn	Australia	Queensland	Brisbane	W. Robinson		A
DEBAbubi	Germany	Bavaria	Munich	K. Hartmann	Stomatitis, tongue ulceration	A
DEBAduqu	Germany	Bavaria	Munich	K. Hartmann	Mild renal insufficiency	A
DEBAgaby	Germany	Bavaria	Munich	K. Hartmann	Dyspnea, cardiac failure	A
DEBAkoob	Germany	Bavaria	Munich	K. Hartmann		A
DEBAmanu	Germany	Bavaria	Munich	K. Hartmann	Kidney failure, respiratory infections	A
DEBAmino	Germany	Bavaria	Munich	K. Hartmann		A
DEBASilv	Germany	Bavaria	Munich	K. Hartmann	Respiratory infections, stomatitis	A
ITPIstas	Italy	Piedmont	Turin	Stefano		A
USCAbogi	USA	Calif.	Oakland	Kitchell	Diarrhea, glaucoma	A
USCAlucy	USA	Calif.	Greenbae	Suranyi	Moderately severe gingivitis	A
USCAmrbl	USA	Calif.	Redondo Beach	Hutson	Wt loss, gingivitis	A
USCApine	USA	Calif.	Portola Valley	L. Wengren		A
USCAsoph	USA	Calif.	Los Angeles	Kitchell	Pernicious anemia, cholangiohepatitis	A
USCAspph	USA	Calif.	Seattle	Kitchell	Asymptomatic (bites)	A
USCAstan	USA	Calif.	Berkeley	Benjamin	Asymptomatic	A
USHImre	USA	Hawaii	Kailua	Jones	Anorexia, depression, dehydration	A
USHIsam	USA	Hawaii	Kaneohe	McBane	Renal failure, cysts, heartworm	A
USIDjake	USA	Idaho	New Plymouth	Norman	Wt loss, gingivitis	A
USINSamy	USA	Ind.	Elkhart	Albate	Dehydration	A
USMEowg	USA	Maine	Portland	Hoyt	Asymptomatic	A
USMNBust	USA	Minn.	St. Paul	U. Minn.	Acute renal failure	A
USWAabk1	USA	Wash.	Seattle	J. Abkovitz		A
USWAabk3	USA	Wash.	Seattle	J. Abkovitz		A
USWAabk4	USA	Wash.	Seattle	J. Abkovitz		A
USWAabk5	USA	Wash.	Seattle	J. Abkovitz		A
USWAflee	USA	Wash.	Lacey	Faler/Fellows	Abcess, lymphadenopathy, respiratory infection	A
USWakikl	USA	Wash.	Lacey	Faler/Fellows	Dental disease, wt loss	A
USWAsea7	USA	Wash.	Seattle	C. Grant		A
ZAWC01	South Africa	Western Cape	Tygerberg	J. Lamprecht		A
ZAWC42	South Africa	Western Cape	Tygerberg	J. Lamprecht		A
USSCboge	USA	S.C.	Charleston	McCullers	Asymptomatic	A (2)
USHIgold	USA	Hawaii	Kaau	Versluis	Chronic respiratory infections	A (3) ^c
USILbb_1	USA	Ill.	Chicago	Houghton	Fever, wt loss, dental abscess	A+B
USILbb_2	USA	Ill.	Chicago	Houghton	Fever, wt loss, dental abscess	A+B
CABCMitc	Canada	British Columbia	Vancouver	Nixon	Asymptomatic	B
DEBAknud	Germany	Bavaria	Munich	K. Hartmann	Stomatitis	B
ITPIimgl	Italy	Piedmont	Turin	Stefano		B
ITTO003PIU	Italy	Tuscany	Pisa	M. Bendinelli	Gingivitis	B
ITTO017PIU	Italy	Tuscany	Pisa	M. Bendinelli	Asymptomatic	B
ITTO019PIU	Italy	Tuscany	Pisa	M. Bendinelli	Asymptomatic	B
ITTO022PIU	Italy	Tuscany	Pisa	M. Bendinelli	Asymptomatic	B
ITTO026PIU	Italy	Tuscany	Pisa	M. Bendinelli	Asymptomatic	B
ITTO045PIU	Italy	Tuscany	Pisa	M. Bendinelli	AIDS	B
ITTO055PIU	Italy	Tuscany	Pisa	M. Bendinelli	Lymphadenopathy, oral abscess	B
ITTO056PIU	Italy	Tuscany	Pisa	M. Bendinelli	Asymptomatic	B
ITTO073PIU	Italy	Tuscany	Pisa	M. Bendinelli	Asymptomatic	B
ITTO076PIU	Italy	Tuscany	Pisa	M. Bendinelli	Lymphadenopathy, gingivitis	B
ITTO082PIU	Italy	Tuscany	Pisa	M. Bendinelli	Asymptomatic	B
ITTO083PIU	Italy	Tuscany	Pisa	M. Bendinelli	Asymptomatic	B
ITTO084PIU	Italy	Tuscany	Pisa	M. Bendinelli	Asymptomatic	B
USALtink	USA	Ala.	Alexander City	Steele	Skin lesions, conjunctivitis	B
USALtom	USA	Ala.	Alexander City	Steele	Wt loss, otitis	B
USCAbudd	USA	Calif.	Redondo Beach	Hutson	Conjunctivitis, salivary gland tumor, wt loss	B
USCArust	USA	Calif.	Campbell	Stevens	Wt loss, urinary tract infection, dental disease	B
USCAsams	USA	Calif.	Campbell	Stevens	Fight abscess, lymphadenopathy	B
USCAtant	USA	Calif.	Alameda	Stewart	Lymphadenopathy, slow-healing abscess	B
USCOtoby	USA	Colo.	Fort Collins	O'Neil	Diarrhea, gingivitis	B
USCTgrif	USA	Conn.	Guilford	Mullin	Wt loss, depression, weakness, bite wounds	B
USGAttrum	USA	Ga.	Doraville	Lloyd	Conjunctivitis, chemosis	B
USILbear	USA	Ill.	Springfield	Schumann	Jaundice, dehydration	B
USILjoey	USA	Ill.	Chicago	Nordwall	Gingivitis	B
USILsque	USA	Ill.	Chicago	Currian		B

Continued on following page

TABLE 1—Continued

Sample code	Country	State/province	City	Source	Clinical symptoms	Subtype ^b
USMAkitt	USA	Mass.	Salem	Ricksgers	Dental disease	B
USMDeliz	USA	Md.	Baltimore	Brunt	Lymphadenopathy, dermatitis	B
USMDsidn	USA	Md.	Baltimore	Brunt	Vomiting	B
USMEjack	USA	Maine	Portland	Hoyt	Facial abscess	B
USMImrle	USA	Mich.	Madison Heights	Houston	Asymptomatic	B
USMOmrki	USA	Mo.	Kansas City	Sundahl	Dental disease	B
USMOpook	USA	Mo.	Kansas City	Lloyd	Sinusitis	B
USMOrasc	USA	Mo.	Kansas City	Lloyd	Diarrhea, gingivitis	B
USNCboom	USA	N.C.	Winterview	Kuhn	Asymptomatic	B
USNCramb	USA	N.C.	Charlotte	Bissell	Gingivitis	B
USORmart	USA	Oreg.	Brookings	Miller	Anorexia, lethargy, wt loss, dehydration	B
USTXkizz	USA	Tex.	Houston	McManis	Asymptomatic	B
USTXoliv	USA	Tex.	Houston	McManis	Asymptomatic	B
USTXtigg	USA	Tex.	Mo. City	McCaine	Gingivitis, stomatitis	B
USTXyefe	USA	Tex.	Mo. City	McCaine	Gingivitis, stomatitis	B
USWI taz	USA	Wisc.	Oregon	Helfand	Gingivitis, stomatitis	B
USNHluca	USA	N.H.	Dover	Sobel	Upper respiratory tract infection	B (1)
USMNnewt	USA	Minn.	Excelsior	DeJong	Asymptomatic	B (5)
DEBA Mike	Germany	Bavaria	Munich	K. Hartmann	Stomatitis	B (6)
USLAgeor	USA	La.	New Orleans	Bohm	Asymptomatic	B (6)
USMDjess	USA	Md.	Baltimore	Brunt	Chronic urinary tract infection	B (6)
USALspuz	USA	Ala.	Alexander City	Steele	Sneezing, fight abscess, fever, depression	B ^c
USVlcar m	USA	Virginia	Roanoke	Quatmann	Depression, wt loss	B ^c
USMEchar	USA	Maine	York	Beekman	Asymptomatic	B (4) ^c
USCAherr	USA	Calif.	Portola Valley	L. Wengren		B (5) ^c
USPAprin	USA	Pa.	Norriston	Wooley	Wt loss, depression, dehydration, ringworm	B (5) ^c
USCOgrou	USA	Colo.	Denver	Batch	Asymptomatic	B (6) ^c
USPAgeor	USA	Pa.	Phoenixville	Wooley	Asymptomatic	B (6) ^c
USMDsilv	USA	Md.	Takoma Park	Kriemelmeyer	Ulceration, stomatitis	B (7) ^c
USALSylv	USA	Ala.	Alexander City	Steele	Periodontal disease	B (8) ^c
CABCblak	Canada	British Columbia	Vancouver	Nixon	Conjunctivitis, dyspnea	C
CABCchec	Canada	British Columbia	Vancouver	Nixon	Stomatitis	C
DEBAfred	Germany	Bavaria	Munich	K. Hartmann	Stomatitis	C ^c
USCOscru	USA	Colo.	Kitridge	Ediger	Wt loss	PCR—
BRSPt2	Brazil	Sao Paulo	Sao Paulo	M. J. D. O. Angelo		PCR— (I—, II—)
BRSPt7/21	Brazil	Sao Paulo	Sao Paulo	M. J. D. O. Angelo		PCR— (Id, II—)
USCAdona	USA	Calif.	Portola Valley	L. Wengren		PCR— (I—, II—)
USCAudc	USA	Calif.	UCDavis	L. Sparger		PCR— (I—, II—)
USCOjeri	USA	Colo.	Arvada	Combs	Asymptomatic	PCR— (Id, II—)
USILpepp	USA	Ill.	Springfield	Nelson	Lethargy, wt loss, vomiting, diarrhea	PCR— (Id, II—)
USMNHast	USA	Minn.	Minneapolis	Amero	Hemolytic anemia	PCR— (Id, II—)
USORkitt	USA	Oreg.	Portland	Jackson	Upper respiratory tract infection	PCR— (I—, II—)
ZAWC03	South Africa	Western Cape	Tygerberg	J. Lamprecht		PCR— (Id, II—)
ZAWC40	South Africa	Western Cape	Tygerberg	J. Lamprecht		PCR— (II—)

^a Abbreviations: USA, United States; U. Minn., University of Minnesota.

^b Unusual heteroduplex mobility is indicated as follows: (1) all heteroduplexes had Rf of ≤ 0.25 ; (2) the fastest heteroduplex was with reference A1 and had an Rf of >0.25 but ≤ 0.5 ; (3) the fastest heteroduplexes were with references A1 and A2 and had Rf of >0.25 but ≤ 0.5 ; (4) the fastest heteroduplex was with reference B1 and had an Rf of >0.25 but ≤ 0.5 ; (5) the fastest heteroduplex was with reference B2 and had an Rf of >0.25 but ≤ 0.5 ; (6) the fastest heteroduplexes were with references B1 and B2 and had Rf of >0.25 but ≤ 0.5 ; (7) the fastest heteroduplex was with reference B1 and had an Rf of >0.5 but ≤ 0.75 ; (8) the fastest heteroduplexes were with references B1, B2, and C1 and had Rf of >0.5 but ≤ 0.75 . Additional amplifications of some of the samples that were negative (PCR—) were conducted under the following PCR conditions: (I) primers DS22/23 in the first round and DS24/27 (56) in the second round with step-up amplification (five initial cycles with annealing at 37°C); (II) primers DS22/27 in the first round and DS-seq3/6 (56) in the second round with step-up amplification as above. Negative (—) reactions and reactions with smaller-than-expected or internally deleted products (d) are indicated.

^c Nucleotide sequence determined in the present study.

ificity and relative sequence divergence were indicated by the mobility of heteroduplexes relative to reannealed homoduplexes. The large majority of samples (85%) fell within a range of approximately 2 to 10% nucleotide divergence from one pair of the subtype references. Outlying samples were compared to each other by HMA, and, in some instances, a representative DNA sequence was determined.

MATERIALS AND METHODS

Cells and DNA isolation. Infection of cats by FIV was determined by Western blot. Blood samples were drawn into heparin- or EDTA-coated tubes. Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Hypaque gradient

centrifugation within 48 h. Aliquots of about 10^6 cells were frozen in liquid nitrogen. High-molecular-weight DNA was prepared either by digestion with sodium dodecyl sulfate (SDS)-proteinase K, followed by extraction with phenol-chloroform and ethanol precipitation (48), or by using the Isoquick Nucleic Acid Extraction Kit (no. MXT-020-100; Microprobe, Garden Grove, Calif.) (8).

PCR. FIV sequences from cat PBMC DNA were amplified in a nested PCR as previously described (56). Briefly, 0.1 to 1 μ g of genomic DNA was added to a first-round reaction of 50 μ l containing 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 to 1.75 mM $MgCl_2$, 1% dimethyl sulfoxide, 1% glycerol, 5 pmol of each primer, and 0.2 mM each deoxynucleoside triphosphate. Standard reaction conditions were 5 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, followed by 30 cycles of 94°C for 15 s, 55°C for 45 s, and 72°C for 2 min during the first round. The time at 72°C was 1 min during the second round. Each round was followed by a final extension at 72°C for 5 min.

First-round primers DS22 (5'-GCTTCATCATTCCTCTCT-3', corre-

sponding to bp 8823 to 8842 of the FIV Petaluma-14 genome, GenBank accession no. M25381) and DS23 (5'-GCGCAAGTAGTGTGGAG-3', corresponding to bp 6785 to 6801 of the FIV Petaluma-14 genome) amplified a 2.0-kb fragment containing the surface and transmembrane portion of the FIV envelope gene. Second-round primers MB1 and MB2 (5'-ATACCAAAATGTGGATGGTG-3', bp 7316 to 7335, and 5'-CAAGACCAATTTCCAGCAAT-3', bp 7866 to 7847 of the FIV Petaluma-14 genome) amplified an internal 550-bp fragment spanning the V3-V4 region of the gp110 surface protein coding sequence. In some instances in which standard amplification conditions (annealing at 55°C) did not yield a product visible by agarose gel electrophoresis and ethidium bromide staining, a "step-up amplification" was carried out with 5 initial cycles at lower annealing temperatures (37 to 45°C), followed by 30 cycles under standard conditions.

Six reference isolates (listed in the legend to Fig. 1) were amplified with primers DS24 and DS27 (56) and cloned into pUC21. For amplification with primers MB1 and MB2, 1 ng of plasmid DNA was added per 50- μ l reaction.

Heteroduplex mobility assay. Five microliters of second-round product from each sample was mixed with 5 μ l of analogous product from a reference sequence or another FIV sample and 1 μ l of 10 $\times$ reannealing buffer (1 M NaCl, 100 mM Tris [pH 7.8], 20 mM EDTA). The mixture was heated to 94°C for 2 min, cooled by placing on ice, mixed with 1/5 volume of 5 $\times$ loading dye (25% Ficoll, 1% Orange G), and loaded onto a 6% polyacrylamide gel (30:0.8 acrylamide:bisacrylamide). Gels (1.5 mm thick) were poured into plates (17.5 by 15 cm) and used in a V16 vertical gel apparatus (GIBCO-BRL) in 1 $\times$ TBE (88 mM Tris-borate, 89 mM borate, 2 mM EDTA) buffer and electrophoresed at a constant 285 V for 2.5 h.

DNA sequencing. Genomic DNA samples containing FIV provirus were subjected to end-point dilution such that a maximum of two of eight samples of a particular dilution were positive after PCR amplification with a modified set of second-round primers suitable for direct sequencing (MB1S, 5'-CAGgtaaaacga cggcagcATACCAAAATGTGGATGGTG-3', and MB2S, 5'-GACaagcagctatgac catgCAAGACCAATTTCCAGCAAT-3', with the sequences in lowercase letters complementary to the universal M13 forward and reverse sequencing primers, respectively). Amplification products thus derived from single-template molecules were purified by agarose gel electrophoresis and affinity column chromatography (no. 28704; Qiagen, Chatsworth, Calif.). Sequencing reactions with fluorescent dye-labeled M13-primers were performed with the Applied Biosystems Inc. thermocycler sequencing kit (Foster City, Calif.), and products were analyzed on an Applied Biosystems model 370 automated DNA sequencer. A nucleotide sequence alignment of the 13 sequences reported here, along with 35 additional sequences from the GenBank database and a matrix of pairwise genetic distances among 51 sequences, are available by anonymous ftp (<ftp://ftp.u.washington.edu/pub/user-supported/mullab/>) or through the World Wide Web (<http://weber.u.washington.edu/~mullab/FIV/>).

Sequence and phylogenetic analyses. DNA sequences included in the phylogenetic analysis were obtained from this study (see Table 1) and from previous GenBank entries (listed in legends to Fig. 1 and 5). Sequences were aligned by using the program CLUSTAL W (59) followed by manual adjustment using GDE (52). Genetic distances were calculated with DNADIST from the PHYLIP software package, using the maximum likelihood method (14). A neighbor-joining tree (46) of the FIV *env* sequences was constructed, and a bootstrap analysis (13) using 1,000 bootstrap replicates was performed to assess the support at each of the internal nodes of the neighbor-joining tree.

To estimate genetic distances from heteroduplex mobilities, a standard curve (see Fig. 3) was generated as follows. Pairs of samples for which DNA sequences were available were annealed, and heteroduplex mobilities were measured under standardized conditions that resulted in strong assay consistency (6). In parallel, the genetic distances between the pairs of aligned DNA sequences were estimated by using a Jukes-Cantor correction for multiple substitutions. Inspection of the plot of DNA distances against HMA mobilities suggested that a sigmoidal curve would best describe the relationship. This curve was fit by performing a linear regression analysis using $\log_{10}$ (DNA distance) as the dependent variable and $\log_{10}$ (1/HMA mobility - 1) as the independent variable. The linear R^2 for the regression was 0.87, and the relationship between DNA distance and HMA mobility (after back-transformation) is described by the following equation: DNA distance = $7.5451(1/\text{HMA mobility} - 1)^{0.29}$. This equation was used to transform observed mobilities into estimated genetic distances that were used by the program FITCH (14) to estimate phylogenetic relationships. Five and four randomized input orders using local and global branch rearrangements, respectively, were used in refining the tree. Analyses using global rearrangements took from 5 to 9 central processing unit-days on a Sun Microsystems SPARCstation 20 and produced minimum sum-of-squares topologies identical to those using local rearrangements.

The numbers of synonymous (d_s) and nonsynonymous (d_n) substitutions per synonymous or nonsynonymous site, respectively, were calculated with the Jukes-Cantor correction for multiple substitutions (23), using the program MEGA (27).

Recombination analysis. Recombinant sequences may often appear on branches at the base of a pair of clades on the phylogenetic tree. An initial screen for putative recombinant sequences was done by (i) visual inspection of the nucleotide alignment, (ii) a computer program (44a) that is a variant of that described by Siepel et al. (51), and (iii) examination of phylogenetic trees with bootstrapping for sequential 127- to 128-nucleotide portions of the total align-

ment. Once the sequences were identified as possible recombinants, they were aligned to 50% consensus sequences of the respective parental subtypes (see Fig. 6) and signature sites were identified.

Nucleotide sequence accession numbers. Sequences reported here were deposited in GenBank under accession no. U57011 to U57023.

RESULTS

HMA of the FIV envelope gene. An epidemiological survey of FIV envelope sequence diversity was conducted by using HMA as a tool for genetic screening (8). Blood specimens were obtained from infected cats as a result of requests sent to veterinarians and researchers. Genomic DNA was isolated, and nested PCR was used to amplify a 550-bp segment of the FIV envelope gene that encodes the viral surface (SU) protein. This fragment spans the V3-V4 region and had not been shown in previous surveys to exhibit length variation (35, 40, 42, 55, 56). The origin, clinical symptoms, and subtype assignments made by HMA (see below) from 98 FIV-infected PCR-positive cats are listed in Table 1. Eleven other samples, from Brazil, South Africa, and the United States, could not be amplified with these primers. In some instances, however, amplification with alternative primer sets resulted in smaller-than-expected products (indicated in Table 1). We were unable to determine if the ultimate PCR failures were due to the absence of viral template, limited sequence variation at the primer binding sites, or possibly extreme divergence from previously described viruses.

For HMA analysis, PCR products were mixed, heat denatured, and reannealed with analogous fragments amplified from three pairs of previously characterized viruses representing subtypes A, B, and C (56) (Fig. 1). Electrophoresis of these fragments revealed differences between the migration rate of homoduplexes (fast) and heteroduplexes (slower, with the rate of migration related to the number of mismatches between the annealed strands). Most of the unknown samples formed heteroduplexes with both references of one subtype that migrated faster than single-stranded DNA (Fig. 1), whereas heteroduplexes formed with the remaining references migrated only into the uppermost quarter of the gel. Three samples for which subtype assignments could be made are shown in Fig. 1A to C, along with a sample from Germany, DEBA_{mick}, the subtype of which could not be assigned (Fig. 1D). DEBA_{fred}, from Germany, formed a fast-migrating heteroduplex with only one of the subtype C references and thus was tentatively assigned as a subtype C outlier. DEBA_{mick} and 14 additional poorly reactive samples were then analyzed against each other by pairwise HMA, the results of which were included in HMA-based phylogenetic analysis (see below).

The majority of FIV envelope sequences examined, 80 of 98 in this survey (Table 1), and 36 of a total of 41 examined to date in other studies (24, 26, 31, 35, 40, 42, 50, 54–56, 60), belong to either subtype A or B. The geographic distributions of subtypes A, B and C were also more widespread than previously found (24, 56). For example, each of these subtypes was found in Munich, Germany, with two subtypes present in cats from the same household (samples kindly provided by K. Hartmann). FIV from cats from Pisa, Italy, were all subtype B, whereas a previous report had found exclusively subtype A (42) (the validity of the previous report's assignments, however, is currently being reassessed [41]). FIV in cats from the United States generally belonged to either subtype A or B, with some cities (e.g., Redondo Beach and Portola Valley, Calif.) and states (e.g., California, Maine) containing both. The preponderance of sequences in the western United States, however, belonged to subtype A, whereas subtype B was most common in the eastern states. The two previously identified subtype C specimens (56) were both from Vancouver, British Columbia,

FIG. 1. Heteroduplex mobility assay of viral DNAs from FIV-infected cats. PCR-amplified FIV envelope gene fragments spanning the V3-V4 region were mixed and reannealed with corresponding reference fragments to determine the subtype: subtype A {lane A1, FIV-Petaluma 14 [USCAPeta14; GenBank accession no. M25381 (35)]; lane A2, FIV-PPR [USCAppr; GenBank accession no. M36968 (40)]}, subtype B {B1, USIL2489 [U11820 (55)]; B2, USMOglwd03B [U02420 (56)]}, or subtype C {C1, CABCPady02C [U02392 (56)]; C2, CABCPbar03C [U02395 (56)]}. Heteroduplexes were separated by electrophoresis through a 6% polyacrylamide gel and stained with ethidium bromide as described in Materials and Methods. Photographs of the ethidium bromide-stained gels are displayed as reversed images. In panel A, references were compared to USIDjake, shown to be subtype A. (B) USWltaz, subtype B. (C) DEBAfred, subtype C outlier. (D) DEBAmick, subtype not determined by HMA.

Canada. Two of three samples obtained from Vancouver for the current survey also belonged to subtype C, while the third was a subtype B virus (samples kindly provided by M. Nixon). The only other subtype C virus found was the outlier DEBAfred from Munich, Germany, while two putative recombinant viruses from Hawaii and Seattle, Wash., contained subtype C-like sequences (see below).

Relationship between envelope sequence subtype and disease. No clear association between subtype and the presence or absence of clinical symptoms was evident. Combining the data from our previous (56) and current study, 36 out of 60 (60%) subtype B-infected cats for which clinical information was available showed symptoms, as did 21 of 26 (81%) of the subtype A-infected cats and all 3 subtype C-infected cats. Thus, subtype B-infected cats were less likely to be symptomatic, although the difference was not statistically significant ($\chi^2 = 3.50$; $P > 0.05$). It should be noted, however, that the majority of samples obtained for these studies were from veterinarians suspecting FIV infection due to symptoms of immunodeficiency. Furthermore, we selected, if possible, samples from symptomatic cats; hence, it is unclear whether one subtype or another is associated with a more rapid progression to disease. Under experimental conditions, only three FIV isolates have to date been shown to consistently induce fatal immunodeficiency disease: an FIV isolate of undetermined subtype, FIV-NCSU1 (12, 65), and rapidly passaged versions of FIV subtype C and B isolates (CABCPady and USCAamel) (10).

Dual infection: natural and experimental. One sample exhibited fast-migrating heteroduplexes with both the subtype A and B reference sequence pairs (Fig. 2). In addition, the sample when self-reannealed (Fig. 2, lane Ho) produced both slow- and fast-migrating bands, indicating the presence of two highly divergent virus populations within the sample. Viral DNA am-

plified in a separate experiment using a subsequently drawn blood sample showed an identical HMA pattern (data not shown). This cat was therefore dually infected with a subtype A virus closely related to FIV-Petaluma14 and a subtype B virus similar to JapanTM2/USIL2489.

Interestingly, a second indication of dual infection with viruses of different subtype was found in samples from specific-pathogen-free cats experimentally inoculated with subtype A viruses from Northern California. Each was subsequently housed in contact with cats previously infected with subtype B viruses (from the Chicago, Ill., area) (data not shown).

Phylogenetic analysis of HMA data. A standard curve was generated that plotted genetic distance versus heteroduplex migration (Rf), using envelope gene fragments from 26 isolates for which sequences were available, and electrophoresis conditions that were optimized (data not shown) for the fragment size examined (Fig. 3). Above a genetic distance of ~20%, which covers the upper range of intersubtype comparisons, heteroduplex mobilities predict genetic relationships with the poorest accuracy. Heteroduplex mobilities were measured for individual comparisons of 99 (from 97 singly infected cats and 1 dually infected cat) *env* sequences from this study against all six subtype reference sequences and for a limited number of pairwise comparisons between unknown, mostly outlying samples. By using genetic distances estimated from the standard curve, a phylogenetic tree was constructed with the FITCH algorithm (14) to show the approximate genetic relationships between the combined set of 105 viral sequences (Fig. 4).

Three major branches containing multiple sequences were observed, corresponding to subtypes A to C, with most samples belonging to subtypes A and B. Two sequences, USHlgold and USSCboge, were unique outliers positioned between the subtype A and subtype C viruses. Two viruses from Canada and

FIG. 2. Dual infection of cat USILbb. Viral DNA from cat USILbb was amplified as described in Materials and Methods and subjected to HMA analysis as described in Materials and Methods and the legend to Fig. 1.

DEBAfred from Germany clustered with subtype C. Subtype B viruses were much more diverse, with many sequences clustered into two groups around the reference sequences USMOglwd and USIL2489 and several additional outliers with branches extending from near the subtype B branches and positioned between the subtype B and C branch points.

DNA sequence analysis of envelope genes of uncertain subtype and identification of inter-subtype recombinant genes. To clarify the genetic relationships among the evident sequence subtypes and genetic outliers identified by HMA, we selected 13 samples for DNA sequencing over the same region used for HMA. We chose sequences that formed fast-migrating heteroduplexes ($R_f > 0.5$) with either none or only one of the subtype reference sequences or with subtype reference sequences from more than one subtype (these invariably were with both subtype B references plus reference C1). An alignment of the deduced amino acid sequences plus the previously published reference sequences is shown in Fig. 5.

Two sequences, USMEchar and DEBAfred, which were shown by HMA to be subtype B and C outliers, respectively, were found to possess a 3-bp deletion at an identical site relative to the other sequences examined, which may have increased their heteroduplex mobilities disproportionately in the comparisons evaluated. These deletions, however, must have occurred independently since their sequences are otherwise not closely related.

USCAherr appeared to be a unique outlier by HMA but by

DNA sequencing was found to be close to the other subtype B viruses USAPrin and USPageor. This difference in the topologies of the HMA tree and DNA sequence tree is due to the fact that not all pairs of sequences were subjected to the HMA; i.e., the mobilities of the USCAherr-USAPrin duplexes were not measured, and neither were the mobilities of the USCAherr-USPageor duplexes. For the HMA tree, then, these distances were implicitly estimated by the Fitch-Margoliash (15) phylogenetic reconstruction method that was used to construct the tree. In contrast, for the DNA sequence tree, these distances were readily estimated from the sequence data.

The USHgold fragment formed the fastest heteroduplexes with the two A references as well as with reference C1. Comparative DNA sequence alignment shows that it contains segments similar to subtype A and C sequences: at 11 signature sites between positions 328 and 419 in the alignment the USHgold sequence agrees only with the C consensus sequence, while sites between 97 and 310 and 442 and 481 were in substantial agreement with the A consensus (Fig. 6). Similarly, the previously published Aomori1 and Yokohama isolates (24) are putative subtype D/B and A/B recombinants, respectively, within the region analyzed (Fig. 6).

The USWAabk2 fragment did not form fast-migrating heteroduplexes with any subtype reference. DNA sequence comparison revealed, however, that it contained adjacent regions of homology to consensus sequences of subtypes A, B, and C (Fig. 6). Although its origin is unclear, this isolate may have arisen from recombination between viruses of these three subtypes.

With the exception of USHgold, one of only two specimens from Hawaii, all of the putative recombinant viruses detected came from geographic regions in which the parental subtypes were also found, consistent with the possibility of dual infection and subsequent recombination.

FIG. 3. Standard curve relating heteroduplex mobility to genetic distance measured by DNA sequence analysis. Envelope gene fragments (550 bp) were PCR amplified from 26 FIV isolates for which DNA sequences were available. Heteroduplexes were generated between 153 of 325 possible combinations, and their mobilities were determined. Genetic distances ranged from 3 to 28%. DNA distance (in percentages) is plotted versus relative heteroduplex mobility (R_f) determined by counting mismatches between sequences. No length variation was encountered; hence introduction of gaps was not required to generate optimized alignments. A curve was fitted to this data (see Materials and Methods), and the equation describing it was used to estimate genetic distances between unknown samples and the reference sequences for use in generating the tree in Fig. 4.

FIG. 4. Phylogenetic analysis of HMA-derived genetic distances. A phylogenetic tree was generated from a matrix with estimated pairwise genetic distances by using the FITCH algorithm with local rearrangements as described in Materials and Methods (sum of squared deviations between observed and reconstructed distances = 5.622). Subtype references are enclosed in boxes. DNA sequences of the corresponding fragments were determined in this study for the 13 underlined sequences.

Phylogenetic analysis of sequenced isolates. An unrooted neighbor-joining phylogenetic tree generated for the 51 available FIV V3-V4 *env* sequences is shown in Fig. 7. Two of the four putative recombinant viruses identified were found to be rooted between subtypes on this tree, while two (Aomori1 and Yokohama) clustered inside subtype B. The frequency distributions of pairwise genetic distances for subtypes A and B and intersubtype comparisons are shown in Fig. 8.

Both subtypes A and B have several clusters that are well supported by bootstrap analysis. Several subtype A isolates [(Dutch6, DUTCH-UTR, DUTCH-K1) and (CA.DIXON, USCAzepy01, USCAhny11)] form clusters, as do the pairs (USCAPETA14, SwissZ1), (EngUK8, WalesUK14), and (USCAlemy01, USCAAtt10A). With the exception of the (USCAPETA14, SwissZ1) cluster, they consist of sequences obtained from cats in relatively close geographic proximity. In contrast, clusters of the subtype B sequences show far less geographic structure. There are three subtype B clusters: B₁, (USALSylv, USCA2546_8, USILbrny03, USIL2517-2, USIL2489, USO-

Klgrl02, Aomori2, JapanTM2, Sendai2, ItalyM2, and ItalyM3); B₂, (USMOglwd, USCOgrou, USMEchar, USTXmtex03, USVAcarm, and USALspuz); and B₃, (USPAgeor, USCAherr, and USPAprin). While the first cluster consists of sequences from Japan, Italy, and the United States, the second and third consist of sequences from geographically distant areas within the United States. When the four putative recombinants were excluded from a neighbor-joining bootstrap analysis (data not shown), bootstrap support substantially increased at several nodes; most notably, support for the node including subtypes D and B increased from 74 to 93% and that for cluster B₁ increased from 66 to 96%.

The phylogenetic relationships within subtype B were carefully scrutinized. Clusters B₁ (when the putative intersubtype recombinants Aomori1 and Yokohama were excluded), B₃, and the core of B₂ (USTXmtex03, USVAcarm, and USALspuz) all showed high levels of bootstrap support (>95%), while several sequences (DEBA Mick, USMDsilv, USMAboy03, and USMOglwd03) came out at intermediate positions

	1	11	21	31	41	51	61	71	81	91	101	111	121	131	141	151	161	170
Fukuoka-D	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Shizuoka-D	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Consensus-D	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
CABCPbar03-C	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
CABCPbar02-C	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
DEBAref-C	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Consensus-C	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
DUTCH-UTR-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
DUTCH-19K1-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Dutch6-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USCAtt_10-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USCAsam_01-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USCALery01-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
CA.DIXON-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USCAzepy01-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USCAhiky11-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Dutch4-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
ItalyM3-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
SwissZ1-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USCAPEta14-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USSCppR-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
SWISS-22-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
ScotUK2-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
WalesUK14-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
FranceWo-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Sendai1-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
EngUK8-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
ItalyM1-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Consensus-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USCAhery-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USAPager-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USPappin-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USMEchar-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USCGrou-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USVAcamp-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USALepus-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USTXmtex03-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USMasboy03-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USMOglwd03-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USMSily-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USOKlgr102-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
FI2546-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USMSily-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Aomori2-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USIL2489-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
FI2517-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Sendai2-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
JapanTM2-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USTibhry03-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Consensus-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
DEBAmick	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Aomori1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USWab2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
USHgold	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Yokohama	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Consensus-A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Consensus-B	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Consensus-C	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Consensus-D	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..

FIG. 5. Alignment of amino acid sequences spanning the third and fourth variable regions of the FIV envelope gene. Amino acids are translated from the region corresponding to nucleotide numbers 7337 to 7846 of the FIV-Petaluma14 genome. Names of the six reference strains used in HMA (see Fig. 1) are underlined. Recombinant and unclassifiable viruses are in the bottom rows, together with consensus sequences for subtypes A, B, C, and D. Previously published sequences, used in Fig. 5 to 8, and their geographical origin are as follows: FIV-14 Petaluma (USCAPeta14) (M25381, M25729) (Petaluma, Calif.) (35, 57); PPR (USCAPpr) (M36968) (San Diego, Calif.) (40); CA.DIXON (L00608) (northern California) (64); CABCPady02, U02392 (Abbotsford, British Columbia, Canada) (56); CABCPbar03 (U02395) and cABCpbar07 (U02397) (Vancouver, British Columbia, Canada) (56); Dutch 4 (X69498) and Dutch 6 (X69499) (Amsterdam, The Netherlands) (42); Dutch19K1 (M73964) and Dutch19K32 (M73965) (Amsterdam, The Netherlands) (50); Dutch UTR 113 (X60725) (Utrecht, The Netherlands) (60); England UK5 (X69495) (London, England) (42); England UK8 (X69496) (Portsmouth, England) (42); FranceWo (L06135) (France) (37); Italy M2 (X69501), and Italy M3 (X69502) (Pisa, Italy) (42); Aomori-1 (D37816), Aomori-2 (D37817), Fukuoka (D37815), Sendai-1 (D37813), Sendai-2 (D37814), Shizuoka (D37811), and Yokohama (D37812) (Japan) (24); Japan TM2 (M59418) (Tokyo, Japan) (30); Scotland UK2 (X69494) (Perth, Scotland) (42); Swiss Z1 (X57002) and Swiss Z2 (X57001) (Zürich, Switzerland) (31); Wales UK 14 (X69497) (Colwyn Bay, Wales) (42); USCAhiky11 (U02402) (El Cerrito, Calif.) (56); USCAlemy01 (U02404) (Fremont, Calif.) (56); USCAsam_01 (U02410) (Albany, Calif.) (56); USCAtt_10A (U02414) (Oakland, Calif.) (56); USCAzepy01 (U02417) (San Francisco, Calif.) (56); USIL2489 (U11820) (Chicago, Ill.) (55); USILbry03 (U02418) (Chicago, Ill.) (56); USMasboy03 (U02419) (Salem, Mass.) (56); USMOglwd03 (U02420) (Kansas City, Mo.) (56); USTXmtex03 (U02422) (Arlington, Tex.) (56); USOKlgr102 (U02421) (Tulsa, Okla.) (56); USIL2517 (Chicago, Ill.) (56); and USCA2546 (northern California) (54).

in the phylogram (Fig. 7), suggestive of possible recombinant origins for these sequences. Consequently, we investigated whether these four sequences were intrasubtype B recombinants, using the three clusters showing high bootstrap support as intrasubtype groupings. USMOglwd03 was the only sequence of the four that showed a pattern suggestive of intrasubtype recombination. The 110 nucleotides at the 5' end of this sequence showed a close relationship to cluster B₁ sequences, and a neighbor-joining phylogenetic analysis grouped this sequence with USIL2546 at a high level of bootstrap support (98%) (these sequences were 3.75% diverged over this region, so this similarity does not appear to be due to an in vitro recombination event [29]). The remaining 401 nucleotides cluster with B₂ sequences, albeit with low bootstrap support (<70%).

None of the HMA outliers chosen for sequencing was located within the A subtype cluster. Two clusters within subtype A were detected in the HMA tree, one with USCAPeta14 and one with USCAPpr. However, since no Dutch viral sequences

were included in the HMA analysis, it is not known whether or not these would have formed a third cluster as seen from sequence analysis. Overall, the A subtype had a range of genetic variation of 0.8 to 14.4% (average of 8.8%).

No objective criteria exist for the assignment of subtypes from an unrooted tree, and attempts to produce a convincing rooted tree by using outgroup envelope gene sequences from FIV strains from puma and pallas cat did not succeed due to mutational saturation (data not shown). As can be seen from the histogram of intrasubtype A and B comparisons shown in Fig. 8A and B, respectively, and the intersubtype comparisons shown in Fig. 8C, the B subtype forms a middle ground that does not appreciably overlap the intersubtype distribution. Therefore, we chose not to divide the B (or the A) subtype into additional subtypes, despite the significant bootstrap support for certain groupings. The B₃+DEBAmick cluster (range of variation, 8.7 to 19.4%; mean, 13.4%) exhibits pairwise distances to the B₁ and B₂ viruses at the upper end of the intra-

	1	11	21	31	41	51	61	71	81	91	101	111	121	130
consA	gaatcaata	gcctattata	acagttgttag	atgggaavav	acagatgttaa	agtttcattg	tcaaagaaca	cagagtcagc	ctggatcatg	gcttagagca	atctcgtcat	ggagacaaag	gaatagatgg	
consB	...C...gn.	..t...c.	.t.v...a	...g.a.g.ta...	g.ca...g.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	
consC	...ggcg	..t...c.	.t...c.	...c.a.g.t...	...v.a...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	
consD	...ggc	..t...c.	.t...c.	...c.r...t...	...g.a...g.	...w...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	
USHigold	...ggc	..t...c.	.t...c.	...a...a.	...a...a.	...g...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	
Signature		A				C			C	A			B	
USWAabk2	...gg	..t...c.	.t...c.	...a...g.	...c.a...ta...	ca...a.	...g...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	
Signature	A	C			B	C				A				
Aomoril	...ggc	..t...c.	.t...c.	...a...g.	...c.g...t...	...a...g.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	
Signature		D									D		B	
Yokohama	...g	..t...c.	.t...c.	...a...g.	...g.g...a.g.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	...a...a.	
Signature	C			B	AB	A	A		A	A	A			
	131	141	151	161	171	181	191	201	211	221	231	241	251	260
consA	gaatggagac	cagattttga	aagtgaag	gtgaaagtat	ctctacagtg	taaatgcaca	aaasaccta	cctttgcaat	gagaagtcca	ggagattatg	gagaagtaac	gggagcttgg	atagagtgtg	
consB	...g	...c	...g	...a	...a	...a	...a	...a	...a	...a	...a	...a	...a	
consC	...g	...c	...g	...a	...a	...a	...a	...a	...a	...a	...a	...a	...a	
consD	at	...g	...c	...r	...a	...r	...c	...t	...c	...g	...r	...c	...a	
USHigold	...g	...c	...g	...a	...a	...a	...a	...a	...a	...a	...a	...a	...a	
Signature		B	A	AA	A	A	A			A	A	BB	A	A
USWAabk2	...g	...c	...g	...a	...a	...a	...a	...a	...a	...a	...a	...a	...a	
Signature		B	A	A	A							CCC		
Aomoril	at	...g	...c	...r	...a	...r	...c	...t	...c	...g	...r	...c	...a	
Signature	DD		D			D				B	B	BB		
Yokohama	...g	...c	...g	...a	...a	...a	...a	...a	...a	...a	...a	...a	...a	
Signature				AA	D	A	A			B	B	BB		
	261	271	281	291	301	311	321	331	341	351	361	371	381	390
consA	gatgtcatag	gaataaatca	aaacttcata	ctgaagcaag	gtttagaatt	agatgttagat	ggaatgttag	ggataatacc	tcactcattg	atcacatgtg	aaaaactcaa	aattgtttcag	gtgcaaatcc	
consB	...t	...ang...	...c.g...	...a...	...a...	...a...	...a...	...a...	...a...	...a...	...a...	...a...	...a...	
consC	...t	...a...	...g...	...a...	...a...	...a...	...a...	...a...	...a...	...a...	...a...	...a...	...a...	
consD	...g	...ct...	...r...	...ty...	...rk...	...a...	...a...	...a...	...a...	...a...	...a...	...a...	...a...	
USHigold	...g	...c	...g	...a	...a	...a	...a	...a	...a	...a	...a	...a	...a	
Signature	C	AAA	A	A	A			C	CC	C	C	D	C	C
USWAabk2	...t	...a...	...ga...	...c...	...a...	...a...	...a...	...a...	...a...	...a...	...a...	...a...	...a...	
Signature	C	C		B				B		C		B		A
Aomoril	...g	...a...	...g...	...c...	...a...	...a...	...a...	...a...	...a...	...a...	...a...	...a...	...a...	
Signature		C	C	B	B			B		B		B		B
Yokohama	...g	...c	...g	...a	...a	...a	...a	...a	...a	...a	...a	...a	...a	
Signature	D		C	B	B			B		B		B		B
	391	401	411	421	431	441	451	461	471	481	491	501	511	
consA	tgtagattgt	accatgtatg	caataataat	gtataattgt	tccttcaaaa	acgggtttac	tatgaaggtg	gatgacotta	ttatgcattt	caatatgaca	aaagctgtgag	aaatgtataa	c	
consB	...g	...t...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	t	
consC	...g	...t...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	t	
consD	...y	...c...	...t...	...y...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	t	
USHigold	...g	...t...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	t	
Signature		CC	C	C		A	A	AA	A	A				
USWAabk2	...g	...t...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	t	
Signature		A	A	A	AA		A	AA	A	A		C		
Aomoril	...g	...t...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	t	
Signature	B					B	B		B					
Yokohama	...g	...t...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	...c...	t	
Signature	B					B	B		B					

FIG. 6. Nucleotide sequence alignment for recombination analysis. Sequences from USHigold, Yokohama, Aomoril, USWAabk2, and DEBAmick were aligned as described in Materials and Methods and are shown with the consensus sequences of all four sequence subtypes. Signature sites are informative sites in which the putative recombinant sequence matches only one of the 50% subtype consensus sequences.

subtype B distribution (Fig. 8B) and greater than intrasubtype A diversity (Fig. 8A). The B₁ cluster (when the putative recombinants, Yokohama and Aomoril, were excluded) formed the tightest cluster, with a range of variation of 2.6 to 9.3% (mean, 5.7%), while, overall, the B subtype had a range of variation extending to 22.4% (mean, 12.8%).

Subtypes C and D are composed of just three and two representatives each, respectively, and form separate clusters branching off the central stem.

The ranges of genetic diversity within versus between subtypes were found to be broader than previously noted for a more limited number of samples (56). It should be reiterated, however, that the sequences from the current study were chosen because they were outliers and hence it is to be expected that this summary inflates the average and range of diversity. Thus, the topology of the HMA tree (Fig. 4) is likely to provide a more representative view of the distribution of subtypes.

Synonymous and nonsynonymous changes across envelope sequence subtypes. In our previous examination of FIV *env* genes (56), we noted differences between the patterns of mutational changes distinguishing HIV and FIV *env* genes. In particular, FIV *env* genes had higher levels of synonymous site changes for a given degree of overall sequence divergence. In the current study, we examined the types of mutational change

as a function of subtype, and we found that the proportion of synonymous substitutions per potential synonymous site is significantly more than the proportion of nonsynonymous substitutions per potential nonsynonymous site in subtypes A, B, and C (Table 2). Furthermore, a twofold higher rate of occurrence of synonymous changes was found in the B clade, as compared to the A clade (Table 2; standard normal deviate, $z = 5.09$; $P < 0.001$), despite similar levels of nonsynonymous site changes ($z = 1.62$; $P > 0.1$). This distinction had been suggested by our previous analysis of just six FIV subtype B sequences, whereas an analogous discriminatory pattern had not been evident for the HIV-1 main group *env* subtypes (56).

DISCUSSION

One of the major theoretical obstacles in generating an effective vaccine against HIV infection is the already large and growing genetic diversity among the viral antigenic targets, in particular the envelope protein. Vaccination trials in small-animal models such as FIV infection of domestic cats seek to mimic this circumstance. However, divergence within the FIV envelope has been less well explored, with four sequence subtypes described to date, that vary by 17 to 26% over the entire gene (24, 56). HMA had been shown to be a rapid and reliable

FIG. 7. Phylogenetic tree analysis of FIV *env* V3-V4 sequences. Included in the 51 viral sequences are 13 viruses from this study (underlined), the 6 reference sequences (boxed) and 32 sequences available from GenBank (see legend to Fig. 5 for accession numbers). Putative intersubtype recombinant sequences are enclosed by rounded rectangles. Branch lengths are drawn to scale. To illustrate consistency, all bootstrap values (which indicate the reproducibility of the particular bifurcation point) greater than 70% obtained with 1,000 replications of bootstrap sampling are shown.

method for classifying HIV-1 *env* genes into sequence subtypes and for inferring intrasubtype diversity (1, 7, 8). To expand the database of FIV *env* gene information, we placed viruses from six countries within subtypes by using HMA of the V3-V4 region of the surface protein coding sequence of *env*. We then refined these relationships by measuring heteroduplex mobilities in order to identify relative genetic outliers for targeted DNA sequence analysis. We found a larger diversity and geographic heterogeneity than previously reported (24, 42, 56). Most striking were viruses from Munich in southern Germany, where subtypes A, B, and C were found.

Despite this expansion of recognized heterogeneity and geographic distribution, the vast majority (>80% overall) of viruses were found to belong to either subtype A or B. None of the genetic outliers identified for DNA sequencing belonged to subtype A, which now appears to be composed of a number of clusters of relatively closely related viruses. Clusters defined by inclusion of Petaluma-14 and PPR were also identified by HMA.

The B subtype was more difficult to define because of the greater breadth of sequence diversity recognized. Nonetheless, three clusters extending from the central stem could be distin-

guished: one (B₁) including those with the prototype sequences TM2 from Japan and USIL2489, a second (B₂) surrounding USMOglwd, and a third (B₃) composed of newly identified sequences including USPAprin and DEBAmick. The distances of the latter group to the other subtype B sequences were at the upper end of the range of intrasubtype-B variation (Fig. 8B) and higher than almost all pairwise distances within subtype A (Fig. 8A). Multiple examples of highly divergent and unique sequences within the broader B subtype were also identified.

Two additional members of the C subtype (current total = 4), and no new members of the D subtype (total = 2) were found.

The results of the phylogenetic analyses of FIV *env* sequences based on HMA and DNA sequence data were in good agreement, with no inconsistencies of subtype designations. Isolates USPAgeor, USPAprin, and USCAherr formed a tight cluster (98% bootstrap support) on the nucleotide sequence tree, whereas they were separate outliers in the HMA tree. However, they were not compared to each other by HMA; thus, the failure to evaluate all possible comparisons by HMA was the likely source of this discrepancy.

Although well-known to be important in the pathogenicity of

FIG. 8. Distribution of genetic distances for 46 FIV *env* V3-V4 nucleotide sequences. Thirty-one FIV-envelope sequences previously determined were obtained from GenBank and aligned with the sequences described here (listed in the legend to Fig. 5, except that USCAhny11 and USCAlemy01 were used; Dutch K32, England UK5, and putative recombinants Aomor1, Yokohama, USH1gold, and USWAabk2 were not included). Pairwise genetic distances were calculated as described in Materials and Methods and arranged in a matrix, and frequencies of distances (increasing at 0.01 intervals) were counted. Genetic distances are plotted versus the number of sequence pairs. Pairwise genetic distances within subtype A are shown in panel A (solid bars), within subtype B in panel B (solid bars), and between subtypes A through D in panel C. Also shown in panel A are the distances of the Dutch cluster to the other sequences and in panel B are the frequencies of distances of the B₃ cluster (DEBA₁mic, USCA₁herr, USPA₁geor, and USPA₁prin) versus other subtype B viruses (stippled bars).

oncoretroviruses (25, 58), recombination between lentivirus genomes was initially slow to be convincingly identified. It has now been shown to occur in both HIV-1 and HIV-2 in geographic regions in which different subtypes have been found to cocirculate (17, 43–45). Several examples of probable recom-

TABLE 2. Synonymous and nonsynonymous mutation rates between FIV envelope subtypes^a

FIV <i>env</i> subtype	<i>n</i>	% Nucleotide divergence			<i>d_s</i> (SE)	<i>d_n</i> (SE)
		Mean	Median	Range		
A	27	8.4	8.6	0.4–14.7	0.201 (0.022)	0.061 (0.005)
B	20	11.3	12.4	2.6–17.0	0.411 (0.035)	0.074 (0.006)
C	3	12.5	11.5	10.1–15.8	0.299 (0.051)	0.109 (0.014)
D	2	4.9	NA	NA	0.090 (0.010)	0.041 (0.031)

^a Mutation rates were calculated as the proportion of synonymous (*d_s*) or nonsynonymous (*d_n*) substitutions per potential synonymous or nonsynonymous site, respectively, after applying the Jukes-Cantor correction for multiple substitutions (23). NA, not applicable.

ination between subtypes were found in this study, through sequence analysis of genetic outliers identified in HMA assays and an analysis of previously published sequences. The parental subtypes of the intersubtype recombinants found were also consistent with the geographic distribution of FIV subtypes; for example, the Yokohama B/D recombinant was found in Japan, where both of the parental subtypes have been found. Although only two viruses from Hawaii were examined, including the USH1gold putative A/C recombinant, the second virus was of the A subtype, while subtype C has also been found in Vancouver, British Columbia, Canada, on the Pacific rim. Presumably, either subtype C viruses or A/C *env* recombinants are circulating in Hawaii or the cat was infected elsewhere and transported to Hawaii.

Recombinants are easiest to identify when they have been generated from two highly divergent viruses infecting the same host. We found such a dual infection by HMA in one of our set of naturally infected domestic cats, in this case with subtypes A and B. Dual infections appear to be rare under natural conditions of exposure and are difficult to detect by sequence analysis (for which either end-point diluted PCR products or plasmid clones are used) unless both subtypes or variants are present in roughly equal quantities or recombinants are found. HMA was also valuable in tracing double infections in our cat colony in which some cats had been experimentally infected with subtype A viruses but housed together with cats infected with subtype B viruses. Over time, some of the subtype A-infected cats became double-infected, in agreement with previously published observations (20, 34). Hence, the immune response ensuing from infection with subtype A FIV does not appear to be protective against subsequent infection with subtype B viruses, at least shortly after infection. This finding is consistent with our hypothesis (56) that subtype B viruses are less apparent to the immune system than subtype A viruses (see below); however, whether prior FIV-B infection interferes with subtype A remains to be determined.

In our previous study of FIV *env* genes (56), we also noted differences between the patterns of mutational changes distinguishing HIV-1 and FIV *env* genes; in particular, FIV *env* genes had evidently achieved mutational saturation at synonymous sites, whereas nonsynonymous changes were similar. This suggested a distinct host-pathogen relationship, with less evidence for immune-driven selection in the feline virus. There was also a trend for this disparity to be subtype specific in cat viruses but not in the human viruses, although too little data was available for the FIV-B subtype to statistically address this hypothesis. In the current study, a striking distinction was indeed found between the evolutionary patterns of the FIV A and B subtypes (Table 2). Substantially more synonymous changes were found in the FIV-B subtype sequences than in

FIG. 9. Relationship of nonsynonymous genetic distances to synonymous genetic distances within FIV subtypes A and B. The number of nonsynonymous substitutions per nonsynonymous site (d_n) is plotted against the corresponding number of synonymous substitutions per synonymous site (d_s) for each within-subtype sequence comparison for subtypes A and B.

FIV-A, while they showed similar levels of nonsynonymous changes.

A plot of d_n versus d_s for both subtype A and B variants (Fig. 9) suggests why this may be the case. Subtype B variants show a pattern of nonsynonymous substitution that appears to plateau with increasing numbers of synonymous substitutions. Such a pattern is consistent with the hypothesis that there is an upper limit to the amount of amino acid variation that can exist within a subtype. It is also apparent in Fig. 9 that, as noted earlier, subtype A viruses have fewer synonymous substitutions than subtype B viruses. This may suggest that subtype A represents a more recent evolutionary lineage; this conjecture is of course contingent on the constancy of generation time and mutation rate across the two subtypes.

We previously reported an absence of symptoms in a smaller sample of cats infected with subtype B viruses, and we therefore hypothesized that subtype B viruses are less pathogenic and immunogenic (49, 56). This hypothesis, though, was not unequivocally supported by analysis of the combined sample set reported here. However, since we were also interested in identifying viruses with a high pathogenic potential, a selection bias occurred. Nonetheless, strong support for this differential disease hypothesis is lacking.

A second hypothesis to explain the evolutionary trends noted is that subtype B viruses may be subject to more structural constraints and/or better adapted to escape host immune surveillance, selecting negatively against amino acid changes, or the number of amino acid substitutions has reached a maximum ceiling consistent with maintaining viral integrity. Any more change may therefore be detrimental to the viruses. A

rigorous test of these contrasting interpretations would require experimental infection of cats with cloned viruses of different subtypes and determination of virus turnover rate (19, 61), rate of mutation fixation, and disease-free interval (18). To date, however, only three viruses have been shown to consistently induce fatal immunodeficiency disease in cats under experimental conditions (10, 12), only one of which has been molecularly cloned (65).

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