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B Epitopes and Selection Pressures in Feline Immunodeficiency Virus Envelope Glycoproteins

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In order to map linear B epitopes in feline immunodeficiency virus (FIV) envelope glycoproteins (Env), a random library of FIV Env polypeptides fused to β-galactosidase and expressed in *Escherichia coli* was screened by using sera from experimentally FIV-infected cats. We mapped five antibody-binding domains in the surface envelope glycoprotein (SU1 to SU5) and four in the transmembrane envelope glycoprotein (TM1 to TM4). Immunological analysis with 48 serum samples from naturally or experimentally infected cats of diverse origins revealed a broad group reactivity for epitopes SU2, TM2, and TM3, whereas SU3 appeared as strictly type specific. To study selection pressures acting on the identified immunogenic domains, we analyzed structural constraints and distribution of synonymous and nonsynonymous mutations (amino acids unchanged or changed). Two linear B epitopes (SU3 and TM4) appeared to be submitted to positive selection for change, a pattern of evolution predicting their possible involvement in antiviral protection. These experiments provide a pertinent choice of oligopeptides for further analysis of the protective response against FIV envelope glycoproteins, as a model to understand the role of antibody escape in lentiviral persistence and to design feline AIDS vaccines.

The feline immunodeficiency virus (FIV) is a member of the lentivirus subfamily of retroviruses (34). Its genomic organization is similar to that of ungulate lentiviruses, such as visna virus, and primate lentiviruses, such as human and simian immunodeficiency viruses (HIV and SIV) (31, 45). Like HIV, FIV is able to infect macrophages, astrocytes, and the CD4 subset of lymphocytes (6, 10, 35). FIV induces a "slow disease" in cats, similar to AIDS in humans, characterized by alterations of the CD4/CD8 T-lymphocyte ratio, susceptibility to opportunistic pathogens, and neurological disorders (for a review, see reference 48).

The envelope glycoproteins mediate important aspects of host-virus relationships and lentivirus pathogenic properties; they govern the cell tropism by interacting at the cell membrane with cellular receptors, and they mediate membrane fusion to allow virus entry and constitute a main target of the immune response. In the case of HIV type 1 (HIV-1), as well as for other lentiviruses, comparisons of amino acid sequences between different isolates have shown that envelope glycoproteins are made of a succession of variable domains which are bordered by conserved domains, most likely determining a conserved spatial framework organization (1, 27). In the case of HIV and SIV, it has been shown that mutations due to the high error rate of polymerases involved in genome replication are subject to positive selection for change; mutations leading to amino acid changes (nonsynonymous) are fixed more frequently in the viral population than synonymous mutations (7, 9, 26, 30, 42). This rarely observed mode of molecular evolution has been suggested to result from an intense selection by the immune system.

Here, we identified linear B epitopes of FIV envelope glycoproteins and analyzed their immunogenicity in the natural host and their sequence variations. By using sera from naturally and experimentally FIV-infected cats and a random library of 30- to 60-amino-acid (aa)-long polypeptides from FIV envelope, fused to β-galactosidase and expressed in *Escherichia coli*, nine immunogenic domains were mapped and characterized as group- or type-specific determinants. Sequence comparisons between FIV isolates revealed that two variable epitopic domains were submitted to positive selection for change. These studies yield detailed information about relationships between lentiviral envelope structure and immunogenicity and provide useful reagents to study antibody escape or design AIDS vaccines.

MATERIALS AND METHODS

Feline sera. Feline sera, obtained from 24 experimentally infected specific-pathogen-free (SPF) cats or 24 naturally infected cats from different regions of France, were kindly provided by A. Moraillon (Ecole Vétérinaire de Maisons Alfort, France) and B. Hurtrel (Institut Pasteur, Paris, France). FIV isolates used for experimental infection were Wo, Me (24), Envnip (18), Villefranche (kindly provided by G. Chappuis, Rhône Mérieux, Lyon, France), and Petaluma (34). SPF cat sera obtained before FIV infection were used as controls. All sera were tested by using FIV Western immunoblot (Idexx, Portland, Maine).

Construction of the λgt11 expression library. Cloning of polymerase chain reaction (PCR)-amplified *env* gene from FIV Wo-infected cat peripheral blood lymphocytes into Bluescript KS plasmid, providing the pKSe construction, has been described elsewhere (33). Random overlapping fragments of pKSe were generated by partial DNase I digestion at 37°C with 0.15 μg of DNase I per ml in 20 mM Tris-HCl (pH 7.4)–1.5 mM $MnCl_2$ for 30 s, yielding fragments of 200 bp average length. DNA fragments were blunt ended with the Klenow fragment of *E. coli* DNA polymerase

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I and ligated with 10-bp *Eco*RI linkers (Pharmacia). Ligation products were digested with *Eco*RI and separated by electrophoresis on a 2% low-melting-point agarose gel (Nusieve). Fragments ranging from 100 to 300 bp were phenol extracted from agarose and ligated with *Eco*RI-digested λgt11 DNA (Promega), in order to obtain recombinant phages that express random *env* fragments as β-galactosidase fusion proteins. The chosen size range allowed minimal immunogenic peptide identification, on the one hand, and detection of antibody-binding sites which requires flanking regions or some local folding, on the other hand. Ligations were packaged by using Packagene (Promega) and plated onto *E. coli* Y1090 on LB medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG). Insert length was checked by PCR using λgt11 primers complementary to the β-galactosidase portion of the λgt11 template [forward, 5′d(GGTGGCGACGACTCCTGGAGCCCG)3′; reverse, 5′d(TTGACACCAGACCAACTGGTAATG)3′]). Eight of 10 randomly picked white clones contained inserts, sized between 120 and 190 bp. The initial library of 10^5 independent clones was then amplified to a titer of 3×10^9 per ml. In situ hybridization of 37 randomly picked plaques with ^{32}P-labelled DNA probes, representing three regions of Wo *env* gene (residues 1 to 977, 978 to 2016, and 2017 to 2562), indicated that the amplified library was equally representative for the entire *env* gene.

Screening procedures and phage-dot. The library was screened by using sera from two cats experimentally infected with FIV Wo isolate. Approximately 3×10^4 PFU from the amplified library were plated on *E. coli* Y1090 (5,000 PFU per plate) and incubated at 42°C for 3 to 4 h. Plates were then overlaid with nitrocellulose filters saturated with 10 mM IPTG and incubated for 3 h more at 37°C. Filters were processed for immunological screening with pooled feline sera diluted 1:300 by using standard methods (defatted milk procedure) (40). Peroxidase-coupled goat anti-cat immunoglobulin G heavy plus light chains (KPL, Gaithersburg, Md.) diluted 1:1,000 were used as second antibodies. Positive reaction was revealed by 4-chloro-1-naphthol (Merck).

Reactive plaques were recovered from agar plugs and submitted to two further cycles of immunological screening for phage plaque purification. Purified plaques were amplified in Y1090 cells, and phage was prepared from bacterial lysate by standard methods (40). A total of 170 positive plaques were detected on the first screening; 108 of these could be purified. Characterization of *env* sequences from a first series of 27 immunologically reactive phages was carried out by direct sequencing of PCR-amplified inserts. Eight inserts (FIV1, FIV2, 9, 35, 44, 90, 147, and 170), corresponding to the nonoverlapping sequences from this first series, were amplified by PCR and ^{32}P labelled by nick translation to be used as probes in situ (40) on the 81 remaining immunologically reactive phages. This strategy reduced the sequencing efforts by defining groups of overlapping inserts and identifying inserts that did not contain a previously sequenced epitope. Thirty-three of these 81 clones were further characterized by sequencing. Of a total of 60 sequenced clones (27 at random in the first series plus 33 chosen in the second series), 48 were finally used for epitope mapping, as phages presenting the same insert (i.e., derived from the same clone) were counted once and phages containing two or more inserts were discarded.

For subsequent phage-dot serological analysis, approximately 100 PFU of isolated phage representing each epitope in 1 μl of SM buffer (40) were plated on a lawn of *E. coli* Y1090 and grown until plaques appeared. A nitrocellulose filter saturated with 10 mM IPTG was placed over the lawn for 3 further h. Immunological screening was performed by using the same protocol as for the initial library screening.

PCR and sequencing. A total of 10 μl of amplified phage lysate was diluted with 60 μl of water and boiled for 10 min. After microcentrifugation, 15 μl of the supernatant was added to 85 μl of PCR mixture containing 50 pM each primer, 10 nM each deoxynucleoside triphosphate and 0.5 U of *Taq* polymerase in a solution of 10 mM Tris-HCl, 50 mM KCl, and 2 mM $MgCl_2$. PCR was carried out for 36 cycles (1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C).

For sequencing, the PCR products were purified by extraction from 1.5% low-melting-point agarose gels (Nusieve). A total of 1 μl of dimethyl sulfoxide and 20 pM either forward or reverse primer were added in a final volume of 10 μl. This mixture was denaturated at 100°C for 12 min and immediately transferred to a liquid nitrogen bath. Next, 1 μl of dithiothreitol (0.1 M), 0.2 μl of Labmix-dGTP (USB), 0.5 μl of [^{35}S]ATP (600 Ci/mM), 0.6 μl of dimethyl sulfoxide, 3.8 μl of water, and 0.2 μl of Sequenase (USB) were added. The samples were incubated at room temperature for 1 min and then at 37°C for 15 min.

Preparation of β-galactosidase fusion protein extracts and Western blot (immunoblot). Env epitopes from clones 9, 27, 44, 51, 54, 124, 133, 154, 157, and 170 were expressed as β-galactosidase fusion proteins by phage λgt11 in lysogenic *E. coli* Y1089 as previously described (19). Crude lysate containing fusion proteins was prepared from 4 ml of IPTG-induced lysogenic cultures by freezing-thawing and sonicating the cell pellet resuspended in 200 μl of TEP buffer (Tris-HCl, 100 mM [pH 7.4]; EDTA, 10 mM; phenylmethylsulfonyl fluoride, 1 mM). Extracts were boiled in Laemmli buffer and resolved on 5 to 10% sodium dodecyl sulfate-polyacrylamide gels (40 μg/well), by using a Mini PII apparatus (Bio-Rad). After protein transfer on nitrocellulose filter and blocking of nonspecific sites by incubation in 3% defatted milk in Tris-HCl (10 mM), NaCl (150 mM), and Tween 20 (0.1%) (TNT buffer), nitrocellulose strips were incubated with cat sera (diluted 1:300) for 2 h at 37°C. Antibody binding was revealed by incubation with peroxidase-coupled goat anti-cat immunoglobulin G (KPL) (diluted 1:1,000) followed by revelation with 4-chloro-1-naphthol (Merck). Controls, constituted by a lysate of *E. coli* Y1089 infected by nonrecombinant λgt11 (antigen control) and by a pool of uninfected SPF cat sera (antibody control), were included in each experiment.

Pepscan analysis. Pepscan was performed to identify precisely the epitopes contained in the broadly reactive SU2 and TM2 regions. Overlapping nonapeptides covering the sequences from 388 to 424 (SU2) and 681 to 711 (TM2) were synthesized in duplicate by Cambridge Research Biochemicals by using the Pin Technology system. Enzyme immunoassay was performed according to manufacturer instructions, by using the sera (diluted 1:200) from five cats infected with different FIV isolates (Wo, Petaluma, Envnip, Villefranche, and Me) and two field-infected cats. Antibody binding was revealed by peroxidase-labelled goat anti-cat immunoglobulin G (heavy plus light chains) and ABTS (Sigma Chemical Co., St. Louis, Mo.) as a substrate. Absorbance values higher than the mean absorbance plus the standard deviation were considered positive.

Computer analysis: structural constraints and nonsynonymous versus synonymous mutations. The *env* sequence of FIV Wo was compared with eight other *env* sequences: two

clones derived from the Petaluma isolate (FIV 34TF10 [United States] [45] and FIV 14 [United States] [31]), FIV PPR [United States] [36], FIV TM2/GVEPX [Japan] [21, 22], FIV Z1 and Z2 [Switzerland] [25], and FIV 19k1 and 19k2 [The Netherlands] [41]). Two profiles were built from the aligned amino acid and nucleotide sequences; the first indicates structural constraints from local amino acid variations, and the second indicates the existence of selection pressures for change by measuring the fixation of nonsynonymous versus synonymous substitutions.

Structural constraints were calculated by summing the values reflecting structural differences (38) between pairs of amino acids present in a given site. To avoid redundancy, especially for closely related isolates, we counted only the pairs of amino acids linked in a minimal spanning tree (37); this tree was derived from the pairwise distances between total amino acid sequences. Next, the measure was normalized so that a maximum value of 1 corresponds to identical amino acids. The profile was smoothed by using a window of 10 sites. Structural constraints were considered weak when values below mean minus two standard deviations (0.85) were observed.

The second index, measuring selective pressures, was calculated as the ratio of the number of nonsynonymous substitutions at nonsynonymous sites to the number of synonymous at synonymous sites (denoted ns_i and s_i, respectively), occurring in a window of 10 sites. Since the number of possible synonymous codons depends on the encoded amino acid, the number of nonsynonymous and synonymous substitutions was divided by the number of possible nonsynonymous and synonymous sites, by using the unweighted pathway method (28). Only the substitutions between amino acid pairs linked in the predefined minimal tree were computed.

RESULTS

Identification of immunoreactive Env peptides. Immunological screening of 3×10^4 clones from the amplified λgt11 Env epitope library, initially containing 10^5 independent clones, was performed by using sera from two cats, experimentally infected with FIV Wo. Forty-eight sequenced inserts from a total of 108 purified immunoreactive clones (see Materials and Methods, screening procedures, for details) allowed the identification of six immunogenic regions of the envelope. The nucleotide sequences of the different immunoreactive inserts and the corresponding peptide sequences are indicated in Table 1. Inside the six immunogenic regions, we mapped clone insert overlaps (43) (Fig. 1). This approach delineated unambiguously eight epitopic domains: five in the external glycoprotein gp100 (SU), designated SU1, aa 253 to 289; SU2, aa 388 to 424; SU3, aa 467 to 492; SU4, aa 508 to 528; and SU5, aa 572 to 606, and three in the transmembrane glycoprotein gp41 (TM), designated TM2, aa 681 to 711; TM3, aa 744 to 788; and TM4, aa 826 to 854. In addition, the peptide sequence 597 to 646, which was defined by clones 20 and 124, overlapped the fifth SU epitope (572 to 606) by 9 aa only, extending in TM by 36 aa and most probably represented a NH_2 terminus TM epitope (TM1). This is supported by immunological screening with other cat sera (cf. results below), which showed differences in reactivity between clones 51 (epitope SU5) and 124 (putative epitope TM1). Similarly, some reactive clones appeared to encompass two epitopes (see, for example, clones 11, 63, and 68 between TM2 and TM3 in Fig. 1). In such a situation, the possibility

TABLE 1. Sequences of the antigenic peptides expressed by cloned recombinant bacteriophages

Viral protein (region) and clone[a]	Reactivity[b]	Nucleotide sequence[c]	Peptide sequence[d]
gp100 SU (1st)			
96	+	727–873	244–291
147	++	735–890	246–296
14	++	747–872	250–290
97	+	750–870	251–289
54	++	755–872	253–290
gp100 SU (2nd)			
93	++	1093–1274	365–424
69	++	1113–1274	372–424
FIV1	++	1122–1274	375–424
12	++	1142–1273	382–424
133	++	1160–1274	387–424
75	++	1163–1274	388–424
gp100 SU (3rd)			
73	++	1377–1536	460–512
148	+	1386–1506	463–502
154	++	1387–1538	463–512
170	+/++	1398–1478	467–492
48	+	1452–1585	485–528
131	+	1452–1599	485–533
136	+	1458–1598	487–532
64	+	1460–1597	488–532
44	+/++	1492–1597	498–532
100	+/−	1502–1637	502–545
158	+	1506–1637	503–545
130	+	1521–1701	508–567
gp100 SU (4th)			
23	+	1654–1820	552–606
103	+	1657–1820	553–606
35	++	1683–1823	562–607
91	++	1700–1822	568–607
51	++	1714–1822	572–607
gp100 SU/gp40 TM (4th)			
42	++	1719–1872	574–623
124	+	1782–1944	595–647
20	+/−	1789–1939	597–646
gp40 TM (5th)			
88	+	1961–2134	654–711
53	+	1299–2153	667–717
163	+	2002–2215	668–738
84	+	2011–2176	671–725
157	++	2019–2176	674–725
FIV2	++	2019–2179	674–726
87	+	2020–2167	674–722
160	+	2020–2140	674–713
108	++	2039–2209	681–736
63	++	2092–2285	698–761
11	++	2097–2282	700–760
68	++	2118–2272	707–757
33	++	2175–2365	726–788
9	++	2226–2366	743–788
168	++	2230–2369	744–789
gp40 TM (6th)			
27	++	2427–2562	810–854
90	+	2475–2562	826–854

[a] Immunogenic regions of envelope are determined by overlapping sequences of reactive clones.
[b] Intensity of immunological reactions was evaluated and graded from +/− (weak) to ++ (strong).
[c] Insert sequences were determined from phage clone DNA. Nucleotide numbering starts from the initiating ATG.
[d] Amino acid positions are numbered from the N-terminal methionine.

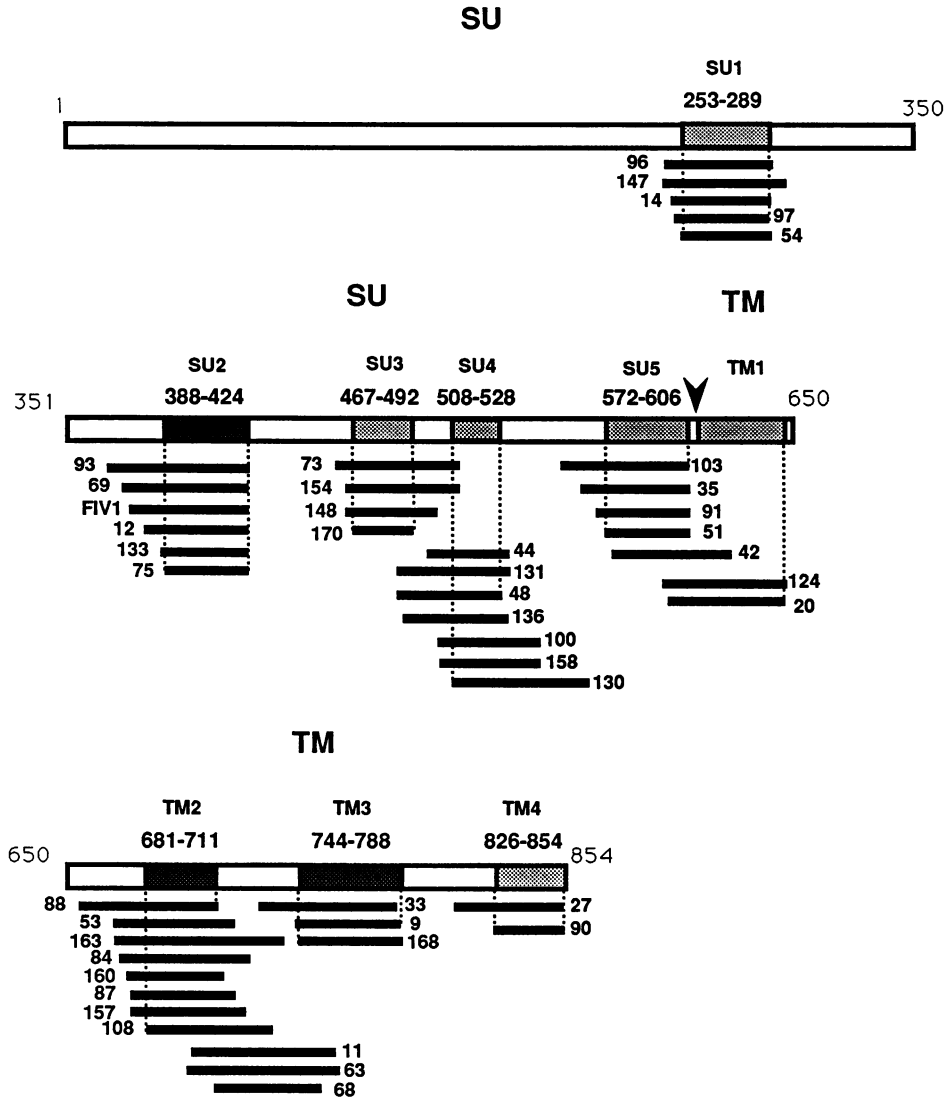

FIG. 1. Location of the epitopic regions on FIV envelope glycoproteins. SU, surface glycoprotein (gp100); TM, transmembrane envelope glycoprotein (gp40). Cleavage site is indicated by a vertical arrowhead. The sequenced clones are represented as horizontal bars with their corresponding identification numbers. Boxes indicate the minimal overlapping sequences, and the corresponding amino acid positions are indicated on top. Darker boxes represent the epitopes with larger reactivity (group-specific determinants).

for an additional antigenic determinant located between regions of overlap remains.

Two regions from the mature Env proteins were not represented at all in clones detected by cat sera (regions located between SU1 and SU2 and between SU2 and SU3 [Fig. 1]). However, several clones covering these regions, nonreactive with cat sera, were detected by using rabbit polyclonal antibodies directed against a synthetic peptide located between SU1 and SU2 (aa 336 to 355) (2) or a mouse monoclonal antibody that mapped between SU2 and SU3 (data not shown). These observations confirmed that these two domains, which appeared free of linear B epitopes for the natural host, were actually represented and stably expressed in the library.

The antibody-binding site of the domains SU2 and TM2 that appeared to be group specific (see below) was further mapped by pepscan analysis by using sera from five experimentally FIV-infected cats and two naturally infected cats. For SU2, four overlapping nonapeptides, corresponding to the sequence WEWRPDFESEK (aa 398 to 408), were reactive. For TM2, three overlapping nonapeptides sharing the pentapeptide QNQFF located between the two cysteine residues 697 and 704 were reactive.

Serological reactivity of identified epitopes in infected cat populations. Sera from 24 cats experimentally infected with five FIV isolates (four isolates from different parts of France: Wo, Me, Villefranche, Envnip; and the original Petaluma strain) and from 24 naturally infected cats (two of these were coinfected by feline leukemia virus) were tested for reactivity with the nine identified antibody-binding domains. All sera had been reactive with the viral structural proteins when tested by commercial Western blot (Iddex [not shown]). A total of 8 uninfected SPF cat sera were used as controls. Bacteriophages clones 9, 27, 44, 51, 54, 124, 133, 154, and 157 containing inserts corresponding to the nine identified domains (Table 1 and Fig. 1) were screened by

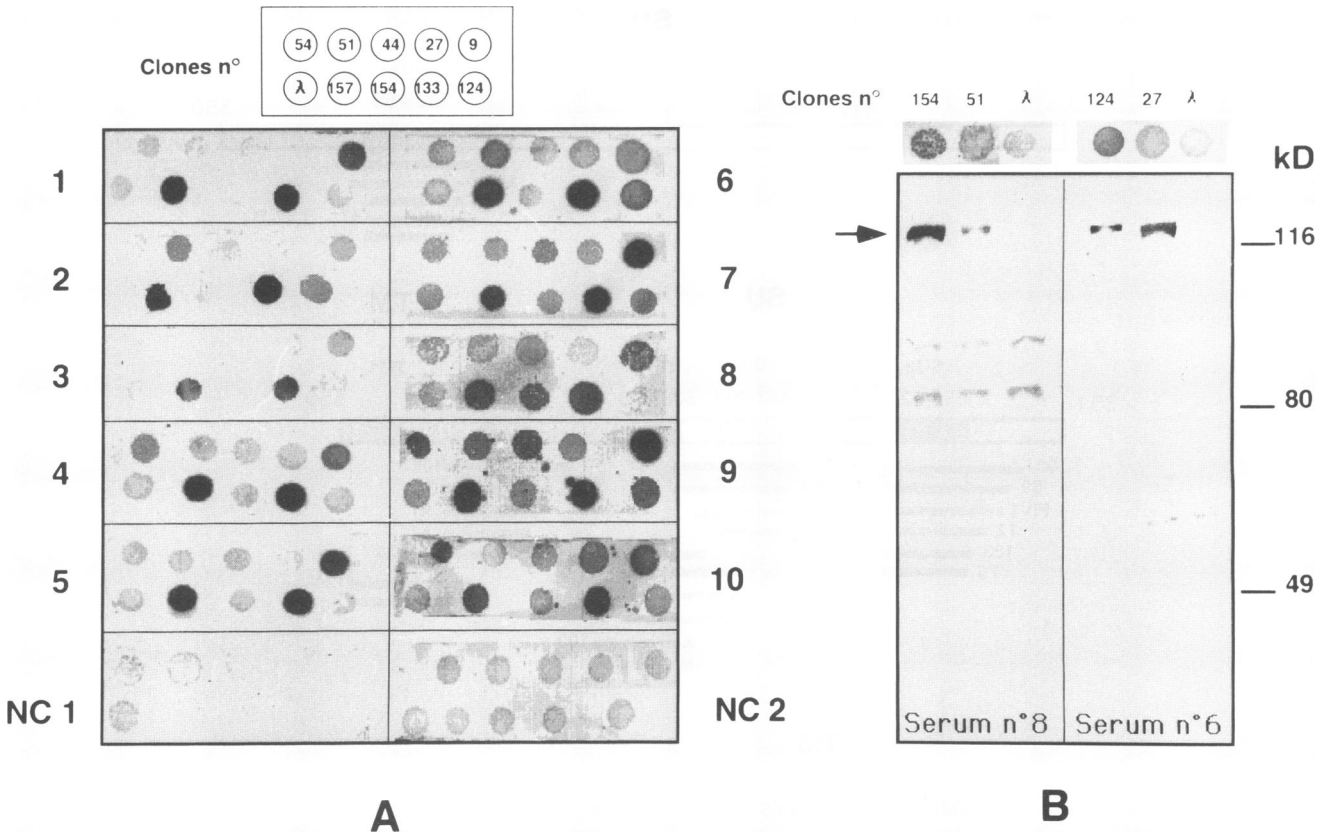

FIG. 2. (A) Phage-dot with cat sera. Each nitrocellulose filter corresponds to one serum sample. Experiments were performed as described in Materials and Methods. Revelation of the reaction with 4-chloro-1-naphthol was continued until the background became visible in the antigen-negative control (nonrecombinant λgt11 [λ] at the lower left of filters). The other dots are blotted from recombinant bacteriophage clones, whose numbers are indicated in the filter representation at the top of the figure. Two experiments are shown (1 to 5 and 6 to 10) with respective negative serum controls from SPF cats (NC1 and NC2). Sera 1 and 7 were from cats inoculated with FIV Villefranche; sera 2, 3, and 10 were from field-infected cats; sera 4 and 9 were from cats inoculated with FIV Envnip; serum 5 was from a cat inoculated with FIV Petaluma; serum 6 was from a cat inoculated with FIV Me; serum 8 was from a cat inoculated with FIV Wo. (B) Western blots were performed as described in Material and Methods by using extracts from *E. coli* infected by lysogenic recombinant phages and by nonrecombinant bacteriophage (λ) as a negative antigen control. Sera are the same as those used in phage-dot 8 and 6 in panel A. At the top are indicated the clone number and the reactions obtained in the corresponding phage-dot assay. The reactive bands corresponding to FIV Env peptides fused to β-galactosidase are visible above the 116-kDa molecular mass marker.

using a direct phage-dot assay (Fig. 2A). Results are reported in Table 2. Clones 133 (SU2), 157 (TM2), and 9 (TM3) showed the broadest reactivity. Conversely, clone 154 (SU3) reacted strongly with serum from the experimentally infected cat (serum 8 in Fig. 2), whose peripheral blood leukocytes were used for FIV Wo *env* gene cloning, and with two other FIV Wo-infected cats sera but did not react with sera of other origins. These results were reproduced by using clone 170 (SU3) expressing a shorter Env peptide. The other epitopes showed variable reactivities (Table 2). Reactivity of infected cat sera observed by phage-dot assay was confirmed by Western blot with recombinant antigens expressed as β-galactosidase fusion proteins in *E. coli* (Fig. 2B).

Selection pressures and epitopic domains. We first computed structural constraints from available protein sequences from nine molecular clones (Fig. 3A). Next, we analyzed corresponding nucleic sequences for both synonymous (s_i) and nonsynonymous (ns_i) change proportions to address the existence and nature of selection that fixed transcription errors in the viral population (Fig. 3B).

These sequences presented the usual pattern of conservative constraints, except for three regions which appeared to follow selection for change ($ns_i/s_i >> 1$), associated with a very low proportion of synonymous changes (Fig. 3B1 and B2). Remarkably, two of these regions induced antibodies in infected cats; they correspond to epitopic domains SU3 and TM4 (Fig. 3C). The third region submitted to selection for change is located in the hydrophobic region corresponding to the putative signal peptide of FIV.

The other epitopic domains did not follow the definition of selection for change pattern. Besides, they showed constraints for structural conservation (Fig. 3A), especially for SU2, TM2, and TM3, that correspond to the three broadly reactive epitopes recognized by the majority of infected cat sera.

DISCUSSION

In the case of HIV, studies are focusing on linear B epitopes on the one hand, typified by the V3 loop, or conformational or glycosylation-dependent epitopes on the

TABLE 2. Serum origins and reactivity of epitopic regions with infected and SPF sera

Serum type and origin[a]	n	No. (%) of reactive sera against following epitopic region (recombinant clone):								
		SU1 (C.54)	SU2 (C.133)	SU3 (C.154)	SU4 (C.44)	SU5 (C.51)	TM1 (C.124)	TM2 (C.157)	TM3 (C.9)	TM4 (C.27)
Experimental										
Wo, i.v.	4	3	4	3	4	4	2	4	4	3
Me, i.v.	6	2	6	0	1	5	4	6	6	4
Petaluma										
i.v.	4	0	4	0	0	0	1	4	4	0
i.c.	2	0	2	0	0	0	0	2	2	0
Villefranche										
i.v.	3	0	3	0	0	0	0	3	3	0
i.c.	2	0	2	0	0	0	0	2	2	0
Envnip, i.v.	3	2	3	0	1	1	0	3	3	0
Natural										
Nantes	10	2	9	0	1	4	4	10	8	1
Alfort	12	5	12	0	1	4	1	12	9	2
FIV + FeLV	2	0	2	0	1	1	1	2	1	0
Total	48	14 (29)	47 (98)	3 (6)	9 (19)	19 (39.5)	13 (27)	48 (100)	42 (87)	10 (21)
SPF	8	0	0	0	0	0	0	0	0	0

[a] Experimental, FIV strains inoculated intravenously (i.v.) or intracerebrally (i.c.). Natural, naturally FIV-infected cats from the veterinary schools of Nantes and Maisons Alfort; FIV + FeLV, cats coinfected with FIV and feline leukemia virus (FeLV).

other hand. Our approach, using peptides from 30 to 60 aa long from FIV envelope produced in *E. coli*, is restricted to the analysis of linear B epitopes. Using a λgt11 epitope expression library, we have defined nine antibody-binding domains within the FIV envelope proteins. Two of these (SU5 and TM4) have been previously identified by using synthetic peptides and enzyme-linked immunosorbent assay (2).

Immunological screening of clones expressing each of the nine immunogenic domains by using 48 sera from experi-

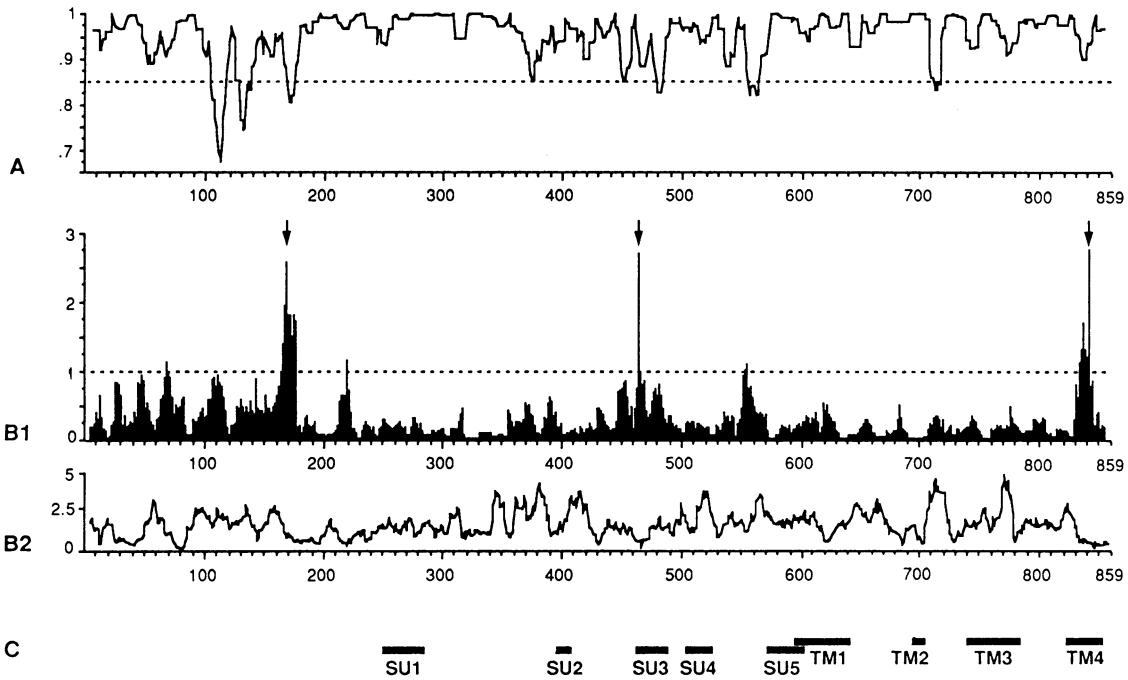

FIG. 3. (A) Profile of structural constraints, based on quantification of amino acid replacements, smoothed by using a window of 10 sites. The maximal value of 1 corresponds to invariant sites. High values correspond to conservative substitutions. Values lower than the threshold of significance (horizontal dashed line at 0.85) indicate high structural change permissivity. (B1) Profile of the ratio of nonsynonymous to synonymous change proportions (ns_i/s_i), smoothed by using a window of 10 sites. Arrows indicate the three higher values, corresponding to selection for change. (B2) Distribution of the synonymous substitutions (s_i). (C) Location of the epitopic domains in SU (SU1 to SU5) and TM (TM1 to TM4).

mentally or naturally infected cats showed that three of them, SU2, TM2, and TM3, were strongly reactive with the totality or the majority of sera. Therefore, these antigenic determinants are good candidates for use in the diagnosis of FIV infection, and pepscan analysis of the SU2 and TM2 domains was performed for a fine identification of amino acids involved in antibody binding. In contrast to these three dominant domains, the epitopic domain SU3 was recognized only by sera from cats infected by FIV Wo, the same strain that was used to construct the epitope library. This strict type specificity was most likely due to the hypervariability of SU3 sequences.

Lentiviral envelope variations have been extensively analyzed. In the case of HIV and SIV, positive selection for change was observed in Env (7, 9, 26, 42). In the case of mature envelope proteins of FIV, we observed that the two domains significantly submitted to selection for change (SU3 and TM4) contained immunogenic sequences (Fig. 3), supporting the hypothesis that this mode of evolution is driven by the host immune response. In addition, such a pattern of evolution is predictive of a protective antiviral immunity directed against these two domains.

We did not find a linear B epitope in the FIV envelope signal peptide, although its C-terminal part is also selected for change. Other mechanisms have to be involved to explain selection of signal peptide variability, including presence of a cytotoxic T-lymphocyte epitope. This hypothesis is supported by the recently described presentation pathway used by signal peptides (16, 47). This could be an important and general issue, as most retroviruses contain long signal peptides with distinct features (11). These features confer unusual functional tolerance to mutations in the case of the HIV-1 signal peptide (11), which is also submitted to selection for change (not shown).

The intracytoplasmic part of the TM of HIV-1 contains a neutralizing epitope, although this region should not be exposed at the virion surface (aa 732 to 746) (5, 8). We also observed a linear B epitope and selection for change in the cytoplasmic domain of the TM of FIV. Since the exact structure and functions of this domain are unknown, the mechanisms underlying such observations remain to be elucidated.

Comparison of B-epitope location in FIV and HIV-1 envelope glycoproteins (14, 17, 23, 29) revealed some similarities. SU5, located at the C terminus of the surface glycoprotein of FIV, which was reactive with 39.5% of infected cat sera, corresponds to the C-terminal epitope of HIV-1 SU, reactive with about 50% of seropositive patient sera (32). An epitope with similar location has been also found in HIV-2, SIV from macaques, and equine infectious anemia virus surface glycoproteins (3, 4).

The hypervariable SU3 domain of FIV, which elicited type-specific antibodies, could correspond to the third variable loop of HIV-1 (V3), which contains the principal neutralizing domain (15, 20, 39). Analysis of the neutralizing effect of anti-SU3 antibodies and research of an associated T-lymphocyte epitope by using the reagents characterized here, will constitute the next important step in the design of a feline AIDS vaccine.

The location of antibody-binding domains within the transmembrane glycoproteins of FIV and HIV-1 (17) appeared even more related; the immunodominant epitope TM2 of FIV, included in a conserved region between two cysteines, corresponds precisely to the immunodominant domain of HIV-1 in the fifth conserved (C5) region (aa 603 to 609) (13, 46). In HIV-1, the conservation of this immunogenic loop is critical for efficient cleavage of the envelope precursor complex (44). However, this immunodominant epitope of the HIV transmembrane protein is not involved in viral neutralization (13). A similar situation could also be expected for TM2 of FIV.

The observed similarities in epitope positions between HIV-1 and FIV are in contrast to the absence of primary structure homologies and the likely differences in the immune responses of humans and cats. Since lentiviruses like HIV and FIV share common ancestors (31, 45), a structural framework has most likely been partly conserved through the evolution of lentiviruses, while the high error rates of viral polymerases lead to loss of sequence similarities. Thus, secondary structure conservation described for all retroviral TMs (12) could also exist to a lesser extent between lentiviral SUs. The identification of the antibody targets within FIV envelope glycoproteins and the production of the corresponding Env peptides, such as described here, can be used for future vaccine design and better understanding of the mechanisms of lentiviral persistence by using FIV infection of cats as an animal model for AIDS.

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