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Insertional Polymorphisms of Endogenous Feline Leukemia Viruses

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The number, chromosomal distribution, and insertional polymorphisms of endogenous feline leukemia viruses (enFeLVs) were determined in four domestic cats (Burmese, Egyptian Mau, Persian, and nonbreed) using fluorescent in situ hybridization and radiation hybrid mapping. Twenty-nine distinct enFeLV loci were detected across 12 of the 18 autosomes. Each cat carried enFeLV at only 9 to 16 of the loci, and many loci were heterozygous for presence of the provirus. Thus, an average of 19 autosomal copies of enFeLV were present per cat diploid genome. Only five of the autosomal enFeLV sites were present in all four cats, and at only one autosomal locus, B4q15, was enFeLV present in both homologues of all four cats. A single enFeLV occurred in the X chromosome of the Burmese cat, while three to five enFeLV proviruses occurred in each Y chromosome. The X chromosome and nine autosomal enFeLV loci were telomeric, suggesting that ectopic recombination between nonhomologous subtelomeres may contribute to enFeLV distribution. Since endogenous FeLVs may affect the infectiousness or pathogenicity of exogenous FeLVs, genomic variation in enFeLVs represents a candidate for genetic influences on FeLV leukemogenesis in cats.

Endogenous feline leukemia virus (enFeLV) sequences are present in the genome of the domestic cat, *Felis catus*. They are homologous to exogenous feline leukemia viruses, which are oncogenic retroviruses capable of inducing both proliferative and degenerative diseases (15, 34). Whereas exogenous FeLVs are transmitted horizontally, endogenous feline leukemia proviruses are part of the germ line and are transmitted from parent to offspring as integral components of chromosomes (5, 20). Endogenous FeLVs are found in wild species of the genus *Felis* closely related to the domestic cat, although they are not present in species from other lineages within the Felidae (4, 56–58). Thus, enFeLVs are believed to have entered the germ line after the initial radiation of lineages in the cat family but before the radiation of domestic cat lineage species (3, 23, 25), although subsequent additional integrations into the germ line may also have occurred (50).

Endogenous FeLV sequences do not by themselves produce infectious virus but readily recombine with exogenous feline leukemia viruses, notably in the *env* region that codes for the viral coat, producing recombinant viruses with altered biological activity and pathogenicity (4, 17, 21, 43, 44, 51, 53, 54, 60). For example, the recombinant subgroup C viruses have been found to induce aplastic anemia (18). Furthermore, portions of the enFeLV *env* region are transcribed and translated in lymphoma and other cell lines (26), producing a truncated envelope protein that inhibits infection by exogenous subgroup B feline leukemia viruses (26). Transcription and translation of enFeLVs have also been demonstrated in tissues from healthy cats, including lymphoid tissue, suggesting a protective role for

enFeLVs in vivo (7, 26). Likewise, inoculation with recombinant subgroup B exogenous FeLV attenuates infection by subgroup A exogenous FeLV (45). By contrast, a protein derived from an enFeLV *env* region was found to facilitate infection by a T-cell-tropic exogenous FeLV (2).

The number of copies of enFeLV per haploid genome has been estimated as 6 to 12 (5, 20, 39, 41, 42), arranged in a nontandem manner (20), while the number of freestanding long terminal repeats (LTRs), believed to have lost their associated coding regions through unequal crossing over during recombination, is ~150 (8). Despite the biomedical impact of feline leukemia viruses and the established capacity of endogenous feline leukemia viruses to recombine with exogenous virus to influence infection and disease progression, the genomic distribution and variation of enFeLVs among domestic cats have not been well characterized. Here, we used fluorescent in situ hybridization (FISH) and radiation hybrid (RH) mapping to determine the chromosomal locations and intraspecies variation of enFeLVs in four domestic cats.

MATERIALS AND METHODS

Probe generation and library screening. To generate probe for screening, long PCR (see below) was performed using the DNA template pKHR-2/λHF60 (13, 33), containing a full-length subgroup B recombinant feline leukemia provirus, using primers GA-GAG-F1 (5'-ATGGGCAAACCTATACTACCC-3') and GA-ENV-R1 (5'-TGGTCGGTCCGGATCGTATTGC-3'). Thermal cycling consisted of an initial 94°C for 4.5 min; 3 cycles of 94°C for 30 s, 66°C for 25 s, and 68°C for 9 min; 5 cycles of 94°C for 30 s, 63°C for 25 s, and 68°C for 9.5 min; 30 cycles of 94°C for 30 s, 60°C for 25 s, and 68°C for 10 min; and a final extension of 68°C for 20 min. This yielded a 7-kb fragment, designated pKHR2-gpe, containing the *gag*, *pol*, and *env* genes, but not the LTRs, of the provirus. The pKHR2-gpe DNA (50 ng) was used to generate probe labeled with [α -³²P]dCTP (New England Nuclear) by random priming (Pharmacia). The probe was eluted through a Nick G-50 Sephadex column (Pharmacia).

A female cat lambda FIX II genomic library (9- to 23-kb insert size; Stratagene) was serially diluted with salt-magnesium buffer for appropriate plating (12 plates; 100,000 PFU/plate) and screened with labeled pKHR2-gpe at low stringency, as described previously (47–49). Positive plaques were rescreened

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until isolated and then plated for extraction of phage DNA (52). Two enFeLV integrations, enFeLV-AGTT (phage 6) and enFeLV-GGAG (phage 16 and 80), have been described (50). A novel enFeLV sequence in phage isolate number 3 was designated enFeLV-ATGC (ATGC for a 4-bp cat genomic sequence at the integration site that is duplicated on either side of the provirus; see below). The enFeLV in phage 18 was designated enFeLV-CTCT, and the enFeLV in phage 94 was designated enFeLV-AGAG.

Distinguishing enFeLV loci. Like other retroviruses (59), FeLV duplicates a small section of the host genome at the integration site. Thus, the 4 bp of feline genomic DNA found immediately upstream of the enFeLV 5' LTR has the same sequence and orientation as the 4-bp feline genomic DNA sequence immediately downstream of the 3' LTR. This duplicated 4-bp feline genomic sequence varies depending on the genomic site at which the enFeLV provirus integrated; thus, we use it to distinguish different enFeLV loci. For example, when the duplicated sequence was AGTT, we referred to the corresponding provirus as enFeLV-AGTT (AGTT for the cat genomic DNA tetramer on either side of the integrated provirus). Likewise, enFeLV-GGAG was used for the enFeLV with the flanking tetramer GGAG. This endogenous leukemia virus integration site label (with an easily remembered acronym) provides a straightforward way of distinguishing among the different enFeLV loci.

DNA sequencing and sequence analysis. For phage that included both enFeLV LTRs, long PCR (see below) was used to isolate each LTR on a separate DNA fragment: phage DNA was amplified using one primer based on the left (FIXII-LA: 5'-GCGGCCGCGAGCTCAATACGA-3') or right (FIXII-RA: 5'-GCGGCCGCGAGCTCAATTAACC-3') phage arm and a second primer based on enFeLV *pol* sequence in either the forward (POL-F8XL: 5'-ACCRA GGRAAACTATAATGCCTGA-3') or reverse (POL-R8XL: 5'-GCCAGCC AGAGAAGGTGTCTAT-3') direction. Thermal cycling consisted of initial heating at 94°C for 2 min; 35 cycles of 94°C for 30 s, 68 (cycles 1 to 5) or 65°C (cycles 6 to 35) for 20 s, and 68°C for 12 min; with a final 30-min extension at 68°C. The single LTR in each PCR-generated segment was then sequenced with primers based on conserved regions of the LTRs, which identified genomic sequence flanking the unique integration site of each enFeLV.

DNA was sequenced using the BigDye Terminator system (Applied Biosystems Inc. [ABI]). Extension products were purified using Centri-Sep spin columns (Princeton Separations) and resolved on an ABI 377 DNA sequencer. The endogenous proviruses were identified by homology to similar sequences in GenBank using BLAST version 2.0 (1).

Long PCR. Long PCR used final concentrations of 800 μ M deoxynucleoside triphosphates, 1.5 mM MgOAc, 800 nM (each) primer, and 2 to 4 U of *rTth* DNA Polymerase XL (ABI) in a 100- μ l total volume. Primers, DNA templates, and product lengths are specified elsewhere in Materials and Methods as appropriate. Ampliwax beads (ABI) were used to ensure a hot start on a GeneAmp PCR System 9700 (ABI). The XL PCR product was purified using Ultrafree-MC centrifugal filter units (Millipore).

FISH. To generate a probe for FISH, long PCR (see above) was performed using as template DNA a phage containing enFeLV-GGAG, which is a full-length enFeLV with no large deletions (50). Primers GAG-F3XL (5'-TTTGG GGGCTCGYCCGGGAT-3') and ENV-R7XL (5'-GGRGMCTAAATGGAAT CATACTT-3') were used to generate a fragment of 7,544 bp, designated 80 (enFeLV-GGAG)-gpe, containing *gag*, *pol*, and *env* proviral regions but not the long terminal repeats. The 80(enFeLV-GGAG)-gpe was used as a probe in FISH.

Following standard procedures (31, 38), metaphase chromosome spreads were prepared from domestic cat primary skin fibroblast cultures and denatured in 70% formamide-2 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 80°C for 90 s. The DNA probe 80(enFeLV-GGAG)-gpe was labeled with biotin-11 dUTP by nick translation, with the resulting probe size being <400 bp. The probe mix (300 to 400 ng of DNA, 10 μ g of salmon sperm DNA in 50% formamide, 10% dextran sulfate, 2 \times SSC) was denatured for 10 min at 78°C and then placed on the denatured cat chromosomes and incubated at 37°C for 24 h. After posthybridization washes, the hybridized biotin-labeled probe was detected using fluorescein isothiocyanate (FITC) conjugated to Avidin DCS (5 mg/ml) (for enFeLV signal) and DAPI (diaminophenylindole) (to label DNA). Fluorescence signal was recorded as grayscale images using an epifluorescence microscope (Axioskop; Zeiss) equipped with a cooled charge-coupled device camera (CE2000; Photometrics), an Apple Macintosh Power Personal Computer, and the Oncor Image system. Grayscale images were merged and pseudocolor was applied using the Oncor Image software. The merged images of DAPI-stained chromosomes and FITC-detected viral signals permitted visualization and cytogenetic localization of integrated FeLV sequences. Twenty metaphase spreads (i.e., 40 copies of each autosome, 20 X chromosomes, and 20 Y chromosomes) were examined for each cat. FITC-detected signal was accepted as evidence for

the presence of enFeLV when signal was detected at a chromosomal site in $\geq 10\%$ of the metaphase spreads.

Statistical analysis. The total number of enFeLV integrations among the eight copies of each autosome (two homologues for each autosome in each of four cats) was divided by the relative physical length of the autosome (30), and a two-tailed *t* test was implemented in Microsoft Excel comparing the number of enFeLVs per unit length among cat autosomes.

RH mapping. RH mapping was performed in a 93-clone domestic cat 5,000-rad RH panel as previously described (35, 36). The primers used for radiation hybrid mapping of the cat genomic regions flanking enFeLV integration sites were as follows: for enFeLV-AGTT, ENFELV6-F (5'-TGGGGGAAAACCTT ACCTTC-3') and ENFELV6-R (5'-CCCCTTCAGTGCATACAACA-3'); for enFeLV-GGAG, ENFELV16-F (5'-GGGCAATTTACCCACAGAGA-3') with ENFELV16-R (5'-CCTCAGCTTTGTCTACGGG-3'); for enFeLV-CTCT, PH18-F1 (5'-TGCAATTTAATGGGGTCTTGG-3') with PH18-R1 (5'-TCTGTG GGGGTGAATTTTTC-3'); for enFeLV-ATGC, ENFELV3-F (5'-TTCCAGG TGTGCAGTATGGA-3') with ENFELV3-R (5'-TAGGGTAGAGGTCCCC AGGT-3'), as well as PH3-F3 (5'-TGCCCATCTCATGGTACCTC-3') with PH3-R3 (5'-TGGCTGGCTTGTAAATGAGGCGGAAGGT-3'). Genomic DNA (ca. 50 ng) extracted using a standard phenol-chloroform protocol (52) or a column-based system (QIAGEN) underwent 35 cycles of amplification by PCR using two oligonucleotide primers (200 nM final concentration) in 1.5 mM MgCl₂, with AmpliTaq-GOLD DNA polymerase (ABI). A hot start at 95°C for 9.5 min was followed by cycles of 20 s at 95°C, 30 s at 60 (7 cycles) or 58°C (28 cycles), and 30 s at 72°C, with a final extension of 2 min at 72°C.

PCR screening of cats for exogenous FeLVs. To rule out the possibility that exogenous FeLV may have infected the cats, two PCR screens were run that would amplify either endogenous or exogenous FeLVs but not both. PCR for endogenous FeLV used primers PFL-F4 (5'-CAGGCTCCCCAGTTGACC-3') and PFL-R4 (5'-AGACCACGAGTCAGATGCAA-3'); PCR for exogenous FeLVs used primers I2-xU3-F1 (5'-ATTTTACAAGGMATGGAAAATTAC-3') and I2-xU3-R1 (5'-TAGTYYAATGAGGCGGAAGGT-3'). Genomic DNA (ca. 50 ng) extracted using a standard phenol-chloroform protocol (52) or a column-based system (QIAGEN) underwent 35 cycles of amplification by PCR using two oligonucleotide primers (200 nM final concentration) in 1.5 mM MgCl₂, with AmpliTaq-GOLD DNA polymerase (ABI). A hot start at 95°C for 9.5 min was followed by cycles of 20 s at 95°C, 30 s at 60 (7 cycles) or 58°C (28 cycles), and 30 s at 72°C, with a final extension of 2 min at 72°C.

Nucleotide sequence accession numbers. The enFeLV sequences discussed in this paper were deposited in GenBank (accession numbers AY364320 to AY364322).

RESULTS

Four male domestic cats were selected for FISH mapping based on the geographic diversity of their origins or on the presence in their genomes of two previously characterized enFeLV proviruses (50) (Table 1). The four cats selected included a Burmese cat (Fca-9), representing a breed of Southeast Asian origin (32); an Egyptian Mau cat (Fca-146), part of a breed likely derived from Egyptian cats (32); a Persian cat (Fca-215), of a breed derived from cats in Iran (32); and a nonbreed cat from the United States (Fca-127) (Table 1).

A 7-kb DNA template of endogenous FeLV, designated 80(enFeLV-GGAG)-gpe, was constructed and used as a probe for FISH. The DNA fragment included the *gag*, *pol*, and *env* regions of a full-length endogenous FeLV (50). The probe template excluded the proviral LTRs in order to preclude detection of ca. 150 copies of enFeLV LTRs without associated coding regions that are present in the genome (6, 8, 40). The presence of exogenous FeLV proviruses in the genomes of the four cats was ruled out by PCR screening (see Materials and Methods).

FISH images of metaphase chromosomes and proviral signals are shown in Fig. 1. Twenty-nine distinct enFeLV autosomal loci were identified across the four cats. The number of autosomal enFeLV loci detected in each cat ranged between 9 and 16 (Table 1 and Fig. 1 and 2). The Persian cat, Fca-215, had 13 autosomal enFeLV loci: 2 distinct integration sites in

TABLE 1. Domestic cats selected for enFeLV FISH

LGD code ^a	Sex ^b	Breed	Geographic origin	Autosomal enFeLVs					
				No. of loci ^d	No. (%) homozygous	Total no. of copies ^e	No. of unique sites ^f	AGTT ^g	GGAG ^g
FCA-9	M	Burmese	Southeast Asia ^c	13	10 (77)	23	5	-/-	-/-
FCA-127	M	Nonbreed	Washington state	9	4 (44)	13	2	-/-	+/-
FCA-146	M	Egyptian Mau	Egypt ^c	16	6 (37)	22	8	+/-	-/-
FCA-215	M	Persian	Iran ^c	13	6 (46)	19	4	-/-	+/+

^a LGD, Laboratory of Genomic Diversity.

^b M, male.

^c Putative geographic origin (32) for cats comprising the breed.

^d Loci are autosomal enFeLV sites present in a cat (counted once whether present in one or both homologues).

^e Total copies are autosomal enFeLVs counted once if present in one homologue (heterozygous) and twice if present in both (homozygous).

^f Unique sites are autosomal enFeLVs present in only one of the four cats.

^g Published (50) PCR screen for individual enFeLVs AGTT and GGAG: +/+, in both homologues; +/-, in one homologues; -/-, missing.

chromosome A2, 2 in chromosome B1, 1 in B2, 1 in B3, 3 in B4, 1 in C1, 1 in D4, 1 in E1, and 1 in F1 (Fig. 1a and 2). The Egyptian Mau cat, Fca-146, had 16 distinct autosomal enFeLV loci: 3 in chromosome A1, 1 in B1, 1 in B2, 2 in B3, 3 in B4, 2 in D2, 1 in D4, 2 in E3, and 1 in F1 (Fig. 1b and 2). The nonbreed cat, Fca-127, had nine distinct autosomal enFeLV loci: two in chromosome A2, one in B1, one in B2, two in B3, two in B4, and one in D4 (Fig. 1c and 2). The Burmese cat, Fca-9, had 13 distinct autosomal enFeLV loci: 2 in chromosome A2, 2 in B1, 1 in B2, 2 in B3, 4 in B4, and 2 in C1 (Fig. 1d and 2).

The number and location of enFeLVs detected by FISH in the four cats are summarized to the left of each cat chromosome in the ideogram (10) in Fig. 2, which positions the 29 distinct autosomal enFeLV integration loci observed across the four cats. Five of these sites (B1q36, B2p15, B3q25, B4q11, and B4q15) were common to all four cats. At 24 other autosomal loci, signal was present only for three (2 sites), two (3 sites), or one (19 sites) of the four cats (Fig. 2); the unique sites limited to one of the four cats are also summarized in Table 1. No enFeLV loci were detected for any of the four cats in chromosome A3, C2, D1, D3, E2, or F2. An X chromosome enFeLV locus was found only in the Burmese cat, Fca-9 (Fig. 1d, 2). Of the 30 enFeLV loci in the X chromosome and autosomes, 10 were telomeric, 1 each in A2p, B1q, B2p, B3q, B4p, B4q, C1p, E1q, E3p, and Xq (Fig. 1 and 2). Only two were centromeric, in chromosomes A2 and B4 (Fig. 1 and 2). The Y chromosomes in all four cats displayed multiple enFeLV loci (Fig. 1f). For each cat, enFeLV was detected at one position in the Yp arm, with signal on two to four positions in the Yq arm. An elongated Yq chromosome arm was present in the Persian cat, Fca-215; additional sites positive for enFeLV were detected in this elongated region of the Y chromosome, possibly suggesting duplication of a Yq fragment (Fig. 1f).

For each cat in Fig. 2, loci at which enFeLV signal was heterozygous, i.e., present in a single chromosome homologue, and homozygous loci at which signal was present in both chromosome homologues in spreads are indicated. The proportion of homozygous sites (Table 1 and Fig. 2) was higher for the Burmese cat, Fca-9 (77% of sites were double), than for the other cats (44% for Fca-127, 37% for Fca-146, and 46% for Fca-215). There were a total of 77 enFeLV copies present at the 29 loci across 144 autosomes (2 homologues for each autosome \times 18 autosomes per cat \times 4 cats). The only autosomal

enFeLV locus that was homozygous in all four cats was the site at B4q15 (Fig. 2). The total number of copies of enFeLV found across the eight copies of each autosome (4 cats \times 2 homologous chromosomes per cat) was greater for the longer chromosomes ($R^2 = 0.119$) (Fig. 3) (30). The eight B4 chromosomes (4 cats \times 2 B4 chromosomes per cat) contained a total of 21 copies of enFeLV (Fig. 2), which was higher than expected based on chromosomal length (Fig. 3) ($P = 0.0063$). Since three of the four cats had been selected for FISH based on known enFeLV proviruses that mapped to chromosome A1 or A2, to exclude ascertainment bias, a separate analysis was run excluding chromosomes A1 and A2 ($R^2 = 0.159$; $y = 89.677x - 0.3812$; $P = 0.0089$ [t test]).

In cat Fca-127, two enFeLV sites in the A2 chromosome homologues were in *trans* configuration: one of the A2 homologues had a single enFeLV copy at A2p24; the other homologue had a single enFeLV copy at A2q11 (Fig. 1c and e). However, ~40% of the metaphase spreads of Fca-127 were trisomic for chromosome A2. In trisomic cells, only the A2q11 signal appeared twice in the same metaphase spread. Thus, A2 trisomy resulted from duplication of the homologue carrying enFeLV at A2q11. Only data from nontrisomic metaphase spreads are included in Fig. 2 and other summaries of the enFeLV FISH results.

An RH panel developed for the domestic cat (35, 36) was used to map the chromosomal location of the unique cat genomic sequence flanking the integration sites of enFeLV-AGTT (50), enFeLV-GGAG (50), and three novel enFeLV proviruses, enFeLV-CTCT, -AGAG, and -ATGC. The genomic DNA flanking the integration site of enFeLV-AGTT mapped to A1q at 1,250.38 centirays (cR_{5000}) between the loci *SEPP1* and *FCAI78*. The DNA flanking enFeLV-GGAG mapped to A2q at 787.91 cR_{5000} between loci *DDC* and *INHBA*. The DNA flanking enFeLV-CTCT mapped to C1p in the interval between loci *GJA4* and *Fc.236810* based on two-point logarithm of the odds scores. The flanks of two endogenous FeLVs, enFeLV-AGAG and -ATGC, had high retention frequencies ($\geq 80\%$) in the RH panel and could not be mapped. High retention frequency is indicative either of a centromeric location or of primers that are amplifying multiple loci (e.g., enFeLV integration within repeat sequences of the feline genome common to multiple chromosomal locations).

Three of the four cats used for FISH mapping were selected in part based upon their carrying previously characterized en-

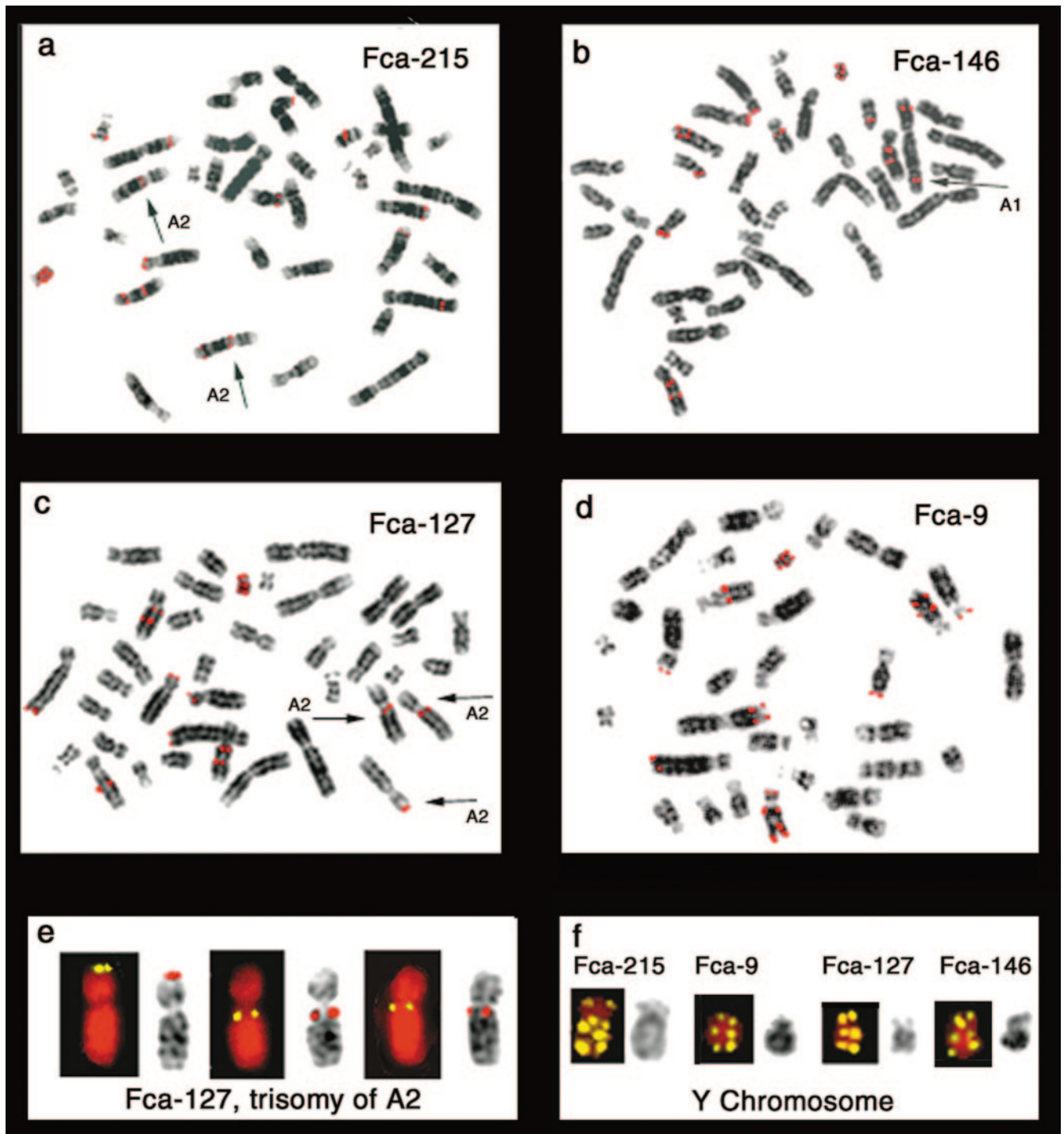


FIG. 1. Chromosomal localization of enFeLVs in metaphase spreads from four domestic cats visualized by FISH. The probe consisted of a segment of enFeLV including *gag*, *pol*, and *env* without LTRs. In panels a to d, hybridization signals (red) are superimposed on reverse DAPI-banded chromosomes. In panels e and f, hybridization signals (yellow) are seen against false-color (red) counterstained chromosomes, which alternate with their DAPI-banded counterparts. (a) Persian cat Fca-215; the arrows point to putative enFeLV provirus enFeLV-GGAG, with signal present at A2q11 in both homologues (50). (b) Egyptian Mau cat Fca-146, with arrow pointing to the putative enFeLV provirus enFeLV-AGTT at A1q26. (c) Nonbreed domestic cat Fca-127; metaphase spread trisomic for chromosome A2. The lowest arrow points to enFeLV in A2p (present in one homologue); the other two arrows point to putative enFeLV-GGAG at A2q11 in the duplicate homologues (see close-up in panel e). (d) The Burmese cat, Fca-9, had the highest proportion of homozygous enFeLV sites (i.e., signal present in both homologues). (e) A2 chromosomes of trisomic metaphase spread of Fca-127 (see also panel c). (f) Proviral enFeLV signal in the Y chromosome shows one site in the Yp arm and two to four sites in the Yq arm of all four domestic cats. In the Persian cat, Fca-215, an elongated Y chromosome included additional enFeLV sites.

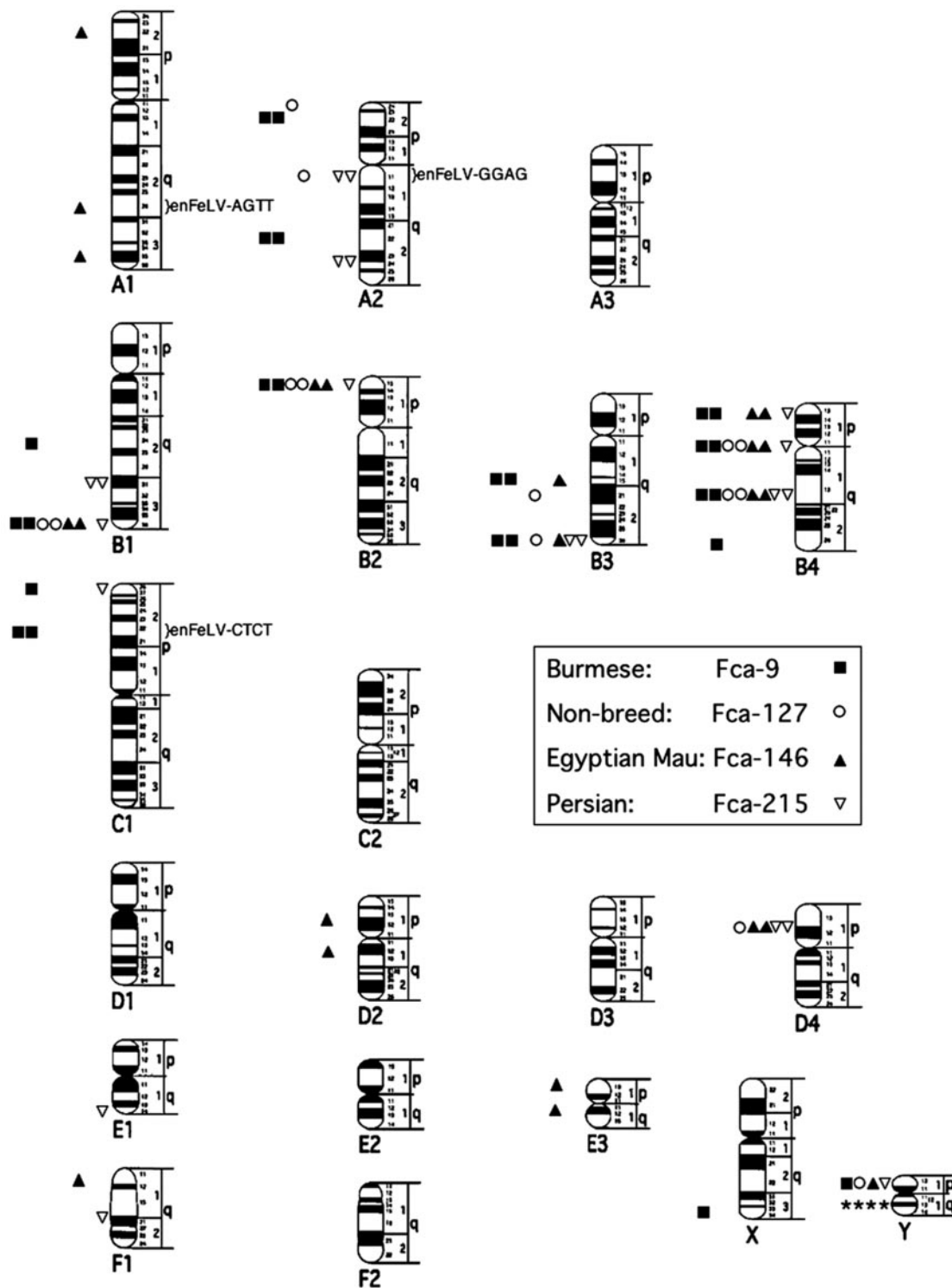


FIG. 2. Ideogram (10) summarizing chromosomal locations of enFeLV copies as determined by FISH in four domestic cats; a distinct icon shape represents each cat. The icons represent loci at which enFeLV was detected in at least 10% of the metaphase chromosome spreads examined. Homozygous loci at which signal was present in both chromosome homologues in an individual are indicated by double icons; single icons indicate signal at only one of the two chromosome homologues (heterozygous). Chromosome A2 in Fca-127 had two enFeLV loci, both heterozygous; the two copies of enFeLV (one at each locus) were in *trans* configuration (i.e., present in different homologues). In all other cases where a cat had two heterozygous enFeLV loci on the same autosome, they were in *cis* configuration (i.e., one homologue had enFeLV present at both loci, and the other homologue lacked enFeLV at both loci). Across 144 total autosomes (4 cats \times 18 autosomes per cat \times 2 homologues for each autosome), signal was present 77 times at 29 distinct autosomal loci resolved by FISH. The asterisks by the Y chromosome indicate that each cat had two to four distinct enFeLV sites in Yq (Fig. 1f). The chromosomal positions of three enFeLV proviruses (enFeLV-AGTT, -GGAG, and -CTCT) were determined by radiation hybrid mapping of the cat genomic DNA flanking the proviral integration site. Their cytogenetic locations, indicated to the right of the chromosomes on the ideogram, were inferred by assuming proportionality between physical locations and genetic distances along the chromosomes (36).

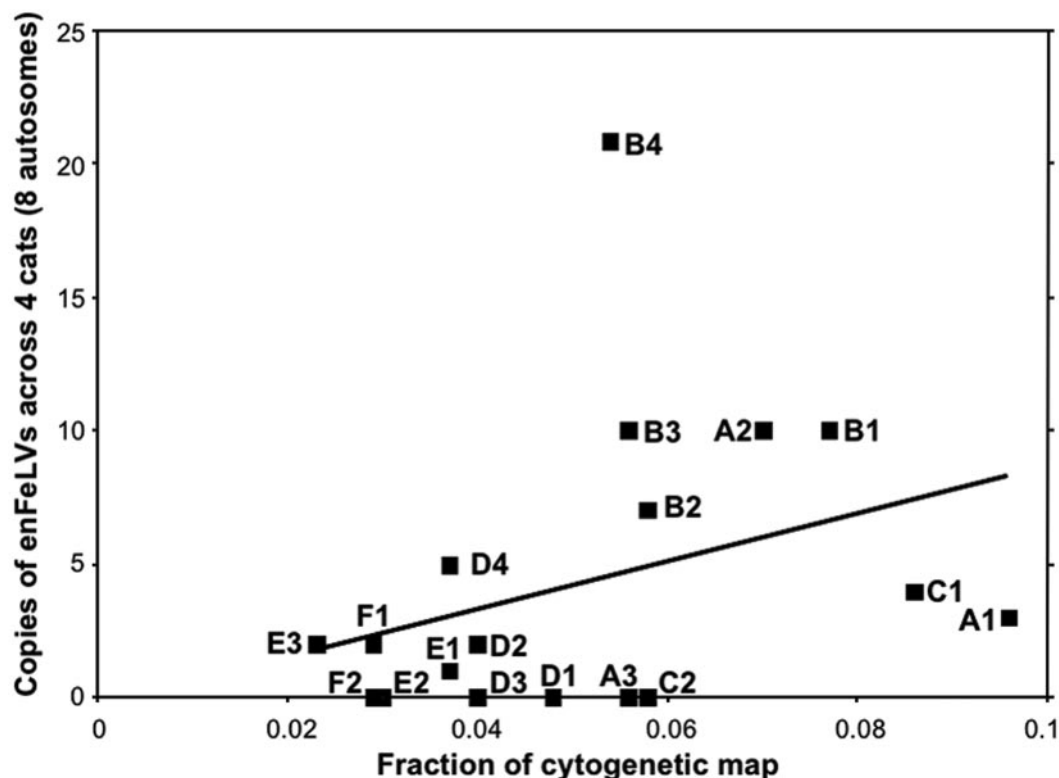


FIG. 3. Copies of endogenous feline leukemia proviruses across four cats versus lengths of autosomes. The *x* axis indicates the relative physical length of each autosome (30). There were a total of 77 copies of enFeLV across the 144 autosomes examined (4 cats \times 18 autosomes \times 2 homologues per autosome) (Fig. 2). On the *y* axis, these copies are tallied across homologous chromosomes (4 cats \times 2 homologues of each chromosome for each cat). The number of enFeLV copies increases with chromosome length ($R^2 = 0.119$; $y = 89.677x - 0.3812$). The eight B4 chromosomes (4 cats \times 2 B4 chromosomes per cat) contained a significantly higher number of copies of enFeLV than expected for an autosome of its length ($P = 0.0063$ [*t* test]).

FeLV proviruses, enFeLV-AGTT and enFeLV-GGAG (Table 1) (50). PCR screens for specific enFeLVs (with one of the primers based on flanking DNA) had demonstrated the presence of enFeLV-AGTT in Fca-146 (in one of the two chromosome homologues) but not in the other three cats (50). Consistent with the PCR results, Fca-146 was the only cat for which FISH detected enFeLV signal in chromosome arm A1q (Fig. 1 and 2), and RH mapping assigned the enFeLV-AGTT flanking region to A1q. Likewise, enFeLV-GGAG had been detected by PCR (with one of the primers based on flanking DNA) in Fca-127 (one homologue) and Fca-215 (both homologues), but not in the other cats (50). Consistent with those results, radiation hybrid mapping assigned the enFeLV-GGAG flank to chromosome A2q, and FISH demonstrated the presence at A2q11 of an enFeLV site found in one homologue of Fca-127 and both homologues of Fca-215 (Fig. 1 and 2). For both enFeLV proviruses, the results of FISH mapping, RH mapping, and previous PCR amplification (with one of the primers based on flanking DNA) were consistent for the four domestic cats. The enFeLV integrations detected by FISH that correspond to the physically mapped enFeLVs are indicated in Fig. 2.

DISCUSSION

FISH mapping allows visualization of the chromosome locations and copy numbers of enFeLVs. FISH detected a total of 29 distinct autosomal enFeLV loci across four cats and

revealed a high degree of previously unexamined insertional polymorphism (intraspecific variation and heterozygous sites). Previous hybridization studies of reassociation kinetics had estimated the number of copies of enFeLV (excluding free-standing LTRs) per haploid genome as 7 to 9 (5), ca. 10 (42), 7 to 10 (40), and 6 to 7 (39). These previous estimates can be compared to FISH results by counting heterozygous sites at an enFeLV locus as one copy and homozygous sites as two copies. There were 23 autosomal enFeLV copies in the Burmese cat, Fca-9; 22 in the Egyptian Mau cat, Fca-146; 19 in the Persian cat, Fca-215; and 13 in the nonbreed cat, Fca-127 (Fig. 2). Across 144 autosomal chromosomes (4 cats \times 18 autosomes per cat \times 2 homologues for each autosome), signal was present a total of 77 times (Fig. 2), an average of 19.25 autosomal enFeLV copies per cat diploid genome. FISH also detected one copy among four X chromosomes and three copies per normal Y chromosome; thus, the total number of enFeLV copies per diploid genome averaged 19.75 in females and 22.5 in males. Half of this diploid average yields 9.87 and 11.25 copies per haploid genome in females and males, respectively, rendering the FISH-based estimate consistent with the previous hybridization studies of reassociation kinetics that had estimated 6 to 10 enFeLV copies per haploid genome (5, 39, 40, 42).

A different approach used previously, restriction enzyme digestion and blot hybridization, had estimated that there are

at least 10 to 12 enFeLVs per cat (the sexes of the cats were not specified) (20). This approach would have identified distinct enFeLV loci without distinguishing homozygous from heterozygous sites, and the results are comparable to the results of FISH if enFeLV loci are counted once regardless of whether they were homozygous or heterozygous. FISH detected 9 to 16 distinct autosomal loci per individual cat (Table 1) (depending on the sex of the cat, 0 to 3 additional sites would be present on the sex chromosomes), consistent with the 10 to ≥ 12 copies previously estimated by restriction enzyme digestion and blot hybridization (20).

FISH detected enFeLV loci in 12 of the 18 autosomes. Longer autosomes (30) had a higher number of copies of enFeLVs ($R^2 = 0.119$), while chromosome B4 contained a disproportionately high number of copies of enFeLVs (Fig. 3). Considerable intraspecific variation was present, with insertional polymorphisms at 28 of 29 autosomal enFeLV loci, and only the B4q15 locus was homozygous in all four cats. The single X chromosome enFeLV site and 19 of the autosomal enFeLV sites were limited to only one of the four cats (Table 1 and Fig. 2), suggesting that additional enFeLV loci would be detected if more cats were examined. The lack of fixation of enFeLV proviruses across domestic cats also suggests that multiple recent integrations or translocations of the proviruses may have occurred within domestic cat genomes (59).

Nine of 29 autosomal enFeLVs were telomeric, as was the single X chromosome enFeLV. Telomeres are dynamic regions of chromosome evolution due to ectopic recombination of subtelomeric regions between nonhomologous chromosomes (12, 28). Nonreciprocal exchanges and duplications among subtelomeric regions are widespread among eukaryotes, and subtelomeric regions are hot spots for the insertion or retention of repeat sequences (12, 28). The high number of telomeric enFeLVs is consistent with a role for ectopic recombination in translocating and perpetuating enFeLVs.

The Burmese cat, Fca-9, had a higher proportion of homozygous enFeLV sites (77%) than the other cats (Table 1), perhaps due to limited outbreeding during development of the Burmese breed (32). The effects of breed differences in enFeLV endowment on breed responses to exogenous FeLV infection are unknown. However, the heterozygous enFeLV sites present in breed cats (Table 1) imply that many enFeLVs are not fixed within breeds; thus, an association between a particular enFeLV and disease outcome may not affect all members of a breed. A study of 134 cats in a household in which FeLV was present found no significant differences in cancer incidence between Abyssinian and Burmese cats (14). While Siamese cats display a predisposition to developing lymphosarcoma (11), this may be unrelated to FeLV (55). One study reported that cats with a solid color in their coats had a higher frequency of FeLV infection (27); another reported that FeLV infection is less common in orange cats than in other cats (46), which was attributed by the authors to behavioral differences ascribed to cats of the orange phenotype (46).

The high number of enFeLV sites present in the Y chromosome is probably due to the absence of recombination over most of its length (9, 19, 37). Although this implies that male cats have a higher copy number of enFeLVs than females, the Y chromosome is largely heterochromatic, with few regions of active transcription. While one study reported a higher fre-

quency of FeLV infection in male cats than in females (27), most studies have found that the incidences of FeLV are similar in the two sexes (16, 22, 24, 46).

For two enFeLV loci, the consistency of results obtained by FISH, radiation hybrid mapping, and PCR screening (with one of the primers based on flanking DNA) established that the combined approaches could determine the locations and copy numbers of different enFeLVs. As the pathogenesis of feline leukemia involves endogenous FeLVs (2, 26, 45), enFeLV locus genotypes could be used to assess the relative influences of different enFeLV proviruses on resistance to and progression of exogenous leukemia virus infection or on the types of subgroup B and C viruses generated through recombination. Among domestic cats exposed to exogenous FeLV, some 72% become infected, while 28% remain uninfected (15). Of those infected, 60% quickly recover and become immune, while 40% are chronically infected (15). Of those chronically infected, 80% die from feline AIDS, anemia, lymphosarcoma, and other diseases associated with FeLV (15). The degree to which these differing outcomes of feline leukemia virus infection are affected by enFeLVs is uncertain, although truncated envelope proteins derived from enFeLV *env* regions inhibit infection by exogenous subgroup B feline leukemia viruses (26) or may facilitate infection by a T-cell-tropic exogenous FeLV (2). Furthermore, subgroup B FeLV, which forms after recombination between exogenous and endogenous FeLVs, may attenuate infection with the nonrecombinant virus (45), while the recombinant subgroup C FeLV induces aplastic anemia in infected cats (18). Thus, differences among cats in their genomic enFeLV dispositions may influence the outcome of exposure to or infection with exogenous leukemia viruses. The genomic variation in enFeLV raises the prospect of implicating these insertional polymorphisms in the development of feline cancers.

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