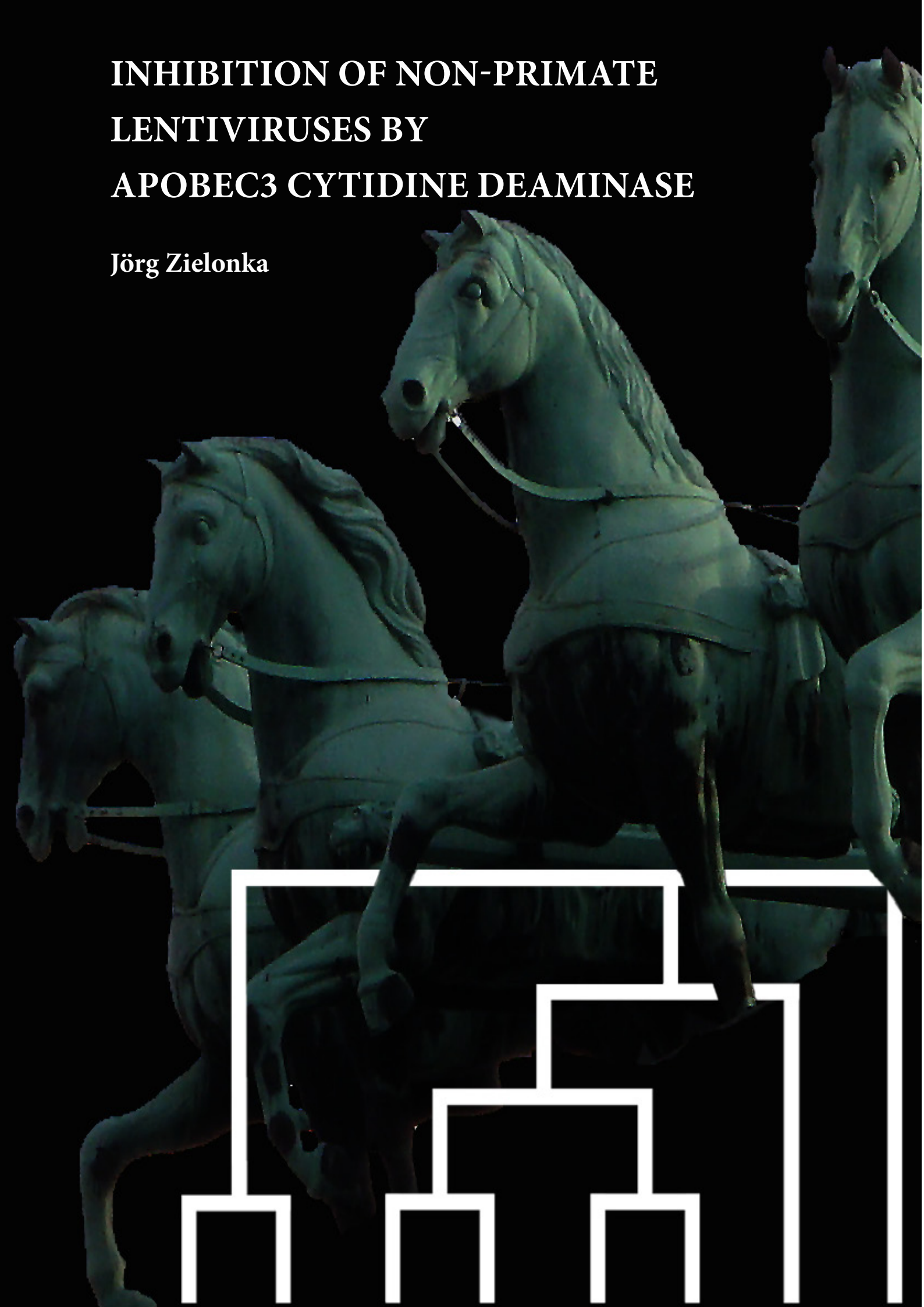
INHIBITION OF NON-PRIMATE LENTIVIRUSES BY APOBEC3 CYTIDINE DEAMINASE

Jörg Zielonka



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Rosa Luxemburg

INHIBITION OF NON-PRIMATE LENTIVIRUSES BY APOBEC3 CYTIDINE DEAMINASE

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Zielonka, J., D. Marino, H. Hofmann, N. Yuhki, M. Löchelt, and C. Münk. Vif of Feline Immunodeficiency Virus from Domestic Cats Protects against APOBEC3 Restriction Factors from Many Felids. J Virol 84:7312-24.

CHAPTER 1

General Introduction

RETROVIRUSES

Retroviruses are RNA viruses, characterized by two essential steps in their replication: reverse transcription of the single-stranded RNA genome into double-stranded DNA and its integration into the host chromosomal DNA.

These membrane-coated viruses carry two identical copies of a single-stranded RNA genome. The retroviral RNA molecules form a dimer stabilized by the dimer linkage structure (DLS) located near the 5'-end of each molecule. All retroviral genomes consist of three major genes: *gag*, *pol* and *env* (FIG. 1). The genomic RNA is capped at the 5'-end by an $_{m7}$G5'ppp5'G$_{m}$p structure, whereas the 3'-end contains a poly (A) tail. At both ends, immediately after the cap at the 5' end and upstream to the poly (A) tail, there are short identical repeated sequences (R). Downstream to the 5'-R lies a unique non-repeated sequence, termed U5, which includes one of the attachment (att) sites required for proviral integration. The U5 region is followed by the primer binding site (PBS), the site of initiation of reverse transcription at which a host tRNA is bound. Encapsidation of the viral genomic RNA into the viral particle requires a specific sequence, called ψ (psi) which is located downstream to the primer binding site. The bulk of sequences that follow are genes encoding viral proteins. They are organized in three major regions: *gag* (group-specific antigen), *pol* (polymerase), and *env* (envelope). The *gag* gene encodes matrix (MA), capsid (CA) and nucleocapsid (NC). The *pol* gene encodes the enzymes: reverse transcriptase (RT), ribonuclease H (RH), and integrase (IN). The *env* gene encodes two envelope proteins, the surface glycoprotein (SU) and the transmembrane glycoprotein (TM). At the 3' end of the genomic RNA, downstream of the *env* gene lies a short polypurine tract (PPT), which is the initiation-site of the plus-strand DNA. The polypurine tract is followed by another unique sequence termed U3. The U3 region contains one of the attachment sites and a number of key *cis*-acting elements for viral gene expression. The 3' copy of the R region, which is followed by the poly (A) tail, is located downstream to the U3 region.

The retroviral replication cycle is a multi-step process (FIG. 2). The major steps are receptor binding and entry, uncoating, reverse transcription, transport of viral DNA into the nucleus, integration into chromosomal DNA, transcription of proviral DNA, splicing of primary transcripts, export of mRNAs to the cytoplasm, translation and post-translational modification, assembly, release, and maturation. The virus binds to host cell receptors via its receptor binding site (RBS), which is located in the surface glycoprotein. Binding causes conformational changes in both the receptor and viral envelope leading to the fusion of virus and cell membranes, internalization of the viral core and uncoating. Reverse transcription takes place in the cytoplasm in a reverse transcription complex which includes the viral RNA, the enzymes (reverse transcriptase, ribonuclease H, and integrase), and the nucleocapsid protein. The viral dsDNA that results from reverse transcription is transported as a pre-integration complex to the nucleus. The integrase cuts the DNA of a host chromosome and integrates the viral DNA into the gap. The integrated provirus replicates synchronously with cellular DNA and each of the daughter cells carries a copy of the integrated viral DNA. The cellular RNA polymerase II starts transcription at the U3-R junction in the 5' long terminal repeat (LTR) and continues to a point located at the R-U5 junction in the 3'LTR. Each full-length transcript is capped and carries a poly (A) tail. RNA transcripts are divided into two populations: spliced and unspliced. Unspliced transcripts serve as genomic

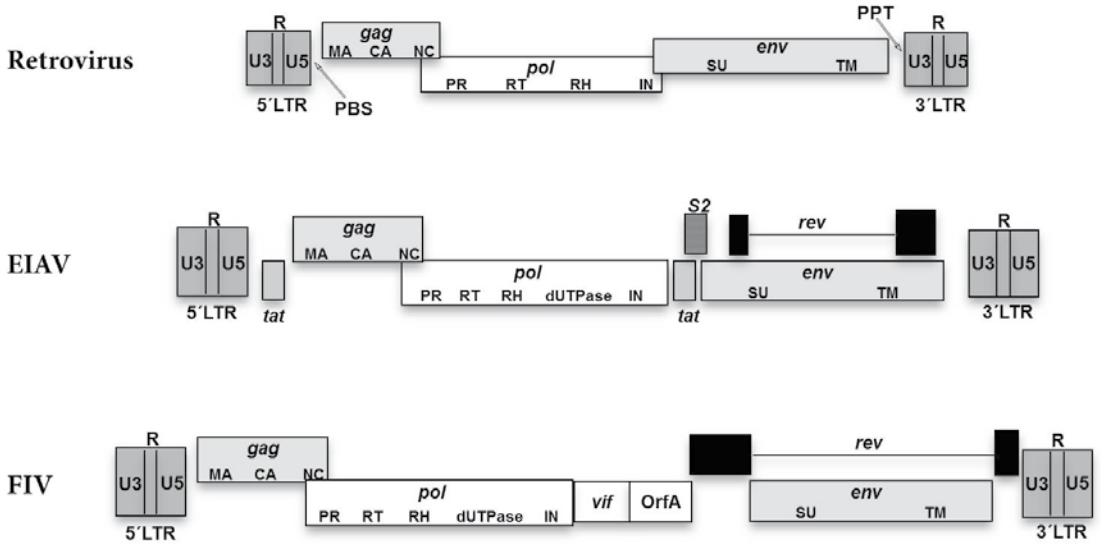

FIG. 1. Retroviral genome organization. The genome organization of a typical retroviral provirus is shown in comparison to the genome organizations of EIAV (*Equine infectious anemia virus*) and FIV (*Feline immunodeficiency virus*).

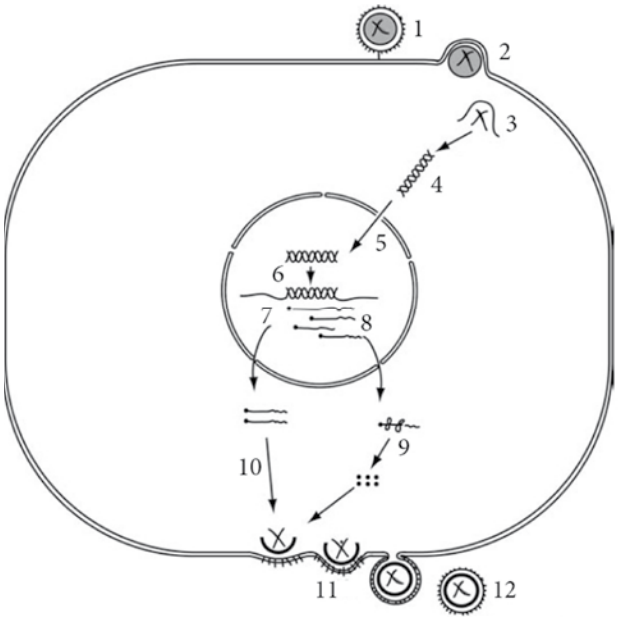

FIG. 2. Scheme of retroviral replication cycle. 1, attachment to the cell surface receptor; 2, internalization; 3, core disassembly and release of viral RNA; 4, reverse transcription into genomic DNA; 5, transport into the nucleus; 6, integration of genomic DNA in the cellular chromosomal DNA; 7, synthesis of RNA transcripts and transport of newly synthesized genomic RNAs to the cytoplasm; 8, transcription of mRNA encoding viral proteins and transport of these transcripts in the cytoplasm; 9, translation of viral proteins; 10, transport of structural viral proteins and genomic RNAs to the sites of assembly; 11, assembly and budding of virions; 12, mature virions. Figure taken from (106). .

RNA, or become translated into Gag, Gag-Pro, and Gag-Pro-Pol precursor polyproteins. The precursor proteins encoded by the *env* gene are translated from spliced mRNAs. The envelope proteins are cleaved by a cellular protease into surface and transmembrane molecules, glycosylated and transported from the golgi complex to the cell membrane. Some retroviruses assemble in the cytoplasm, but most retroviruses form immature particles on the inner surface of the plasma membrane. This process is driven primarily by the Gag precursor protein. Three domains of Gag are essential for assembly: membrane binding domain (M), interaction domain (I), and late assembly domain (L). The membrane binding domain located at the N-terminus of Gag precursor within the MA part is responsible for Gag binding to the cellular membrane. The interaction domain is mainly located within the nucleocapsid part, binds to the viral genomic RNA and mediates the formation of the RNA dimer. The late assembly domain binds host cell factors that are involved in the budding process. Viral proteins together with viral genomic RNA assemble at the cell periphery into immature viral particles that are released from the cell by budding of the plasma membrane. Budding of retroviruses requires cellular ESCRT proteins. Infectious virions are generated outside the cell by a maturation process with proteolytic cleavage of Gag and Gag-Pol by the viral protease. After Gag cleavage the matrix, the capsid and the nucleocapsid proteins are released, while the cleavage of Pol forms the viral enzymes.

LENTIVIRUSES

The genus Lentivirus of the *Retroviridae* family is characterized by its morphology, accessory genes, which are not present in other retroviral genomes, and by a biphasic course of viral gene expression. Six subgroups of lentiviruses (primate, feline, ovine, bovine, equine, and lagomorph lentivirus) were identified, reflecting the mammalian hosts with which they are associated. In 2007 the last group of lagomorpha lentiviruses was discovered in the European rabbit (*Oryctolagus cuniculus*) (45). It was the first characterized endogenous lentivirus, which was termed *rabbit endogenous lentivirus type K* (RELIK). This virus is >7 million years old and thus provide evidence for an ancient origin of the lentiviruses.

The lentiviruses are naturally restricted in their host range, but natural cross-species infections have been detected for SIV (*Simian immunodeficiency virus*) in different monkey lineages (32, 39). In addition, experimental infections of Baboons with HIV-2 (*Human immunodeficiency virus*-2) and chimpanzees with HIV-1 were reported (57, 58, 72), but transmissions of SIV and HIV to non-primates were unsuccessful.

All lentiviruses have the ability to replicate in macrophages. HIV and SIV also infect $CD4^+$ lymphocytes. Primate lentiviruses use a receptor (CD4) and a chemokine co-receptor (mainly CCR5 and CXCR4) for entering these target cells. CXCR4 is also used by the non-primate lentivirus FIV (*Feline immunodeficiency virus*) as entry co-receptor (22, 75, 94, 108).

In addition to the *gag*, *pol*, and *env* structural genes present in all retroviruses, the majority of lentiviruses have additional genes like *tat* (transactivator of transcription) and *rev* (regulatory virus factor). Tat is essential for the replication of HIV, SIV and EIAV (*Equine infectious anemia virus*) as a general activator of transcription. All three lentiviruses have long terminal repeats with low basal activity that is strongly induced by their respective Tat proteins. Rev protein regulates the export of unspliced and single spliced viral RNA containing a Rev response element (RRE) from the nucleus to the cytoplasm. Except EIAV and RELIK, all members of the lentiviral family possess a *vif* (virus infectivity factor) gene. A deletion of the *vif* gene results in dramatically reduced viral replication (30, 47, 90, 98). Four accessory genes are specific for primate lentiviruses: *nef* (negative regulatory factor), *vpr* (viral protein R), *vpx* (viral protein X), and *vpu* (viral protein U). Nef down-regulates the CD4 and MHC class I and II proteins expression at the host cell surface of infected cells. Vpr of HIV-1 facilitates a G_2 cell-cycle arrest (38, 44, 76) and counteracts not identified cellular proteins. Other primate lentiviruses like HIV-2 contain a *vpx* gene which is important for infection of resting monocytes (7, 91). Vpu enhances virion production by counteraction of the cellular tetherin protein (69, 104) and mediates the degradation of CD4 by the ubiquitin-conjugating pathway (86, 109). Exclusively non-primate lentiviruses include a domain for deoxyuridine triphosphatase (dUTPase) in the *pol* region. dUTPase catalyzes the conversion of dUTP to dUMP and inorganic pyrophosphate (PPi). The coding region of dUTPase may have been lost during

the evolution of primate lentiviruses. Alternatively, one viral lineage may have acquired dUTPase sequences from another viral lineage by a recombination event (5). The loss of dUTPase in the evolution could have forced a replication of primate lentiviruses in activated cells (lymphocytes) whereas non-primate lentiviruses predominantly replicate in resting cells with little potential to divide (macrophages). Primate lentiviruses are able to incorporate uracil DNA glycosylase through binding to *vpr* (63). Both dUTPase and uracil DNA glycosylase reduce misincorporation of dUTP in reverse transcription products (17).

EIAV

The *Equine infectious anemia virus* is the smallest and simplest characterized lentivirus. Beside the three major genes (*gag*, *pol*, and *env*), the EIAV genome contains three open reading frames encoding Tat, Rev, and S2 proteins (FIG. 2). The S2 gene is unrelated to other lentiviral genes and is encoded as a distinct reading frame in the *pol-env* intergenic region. The function of the small S2 protein (8 kDa) remains unclear. Mutation studies indicate that S2 is dispensable for *in vitro* replication (53), but it is important for *in vivo* infection (52). S2 is detectable in the cytoplasm where it potentially interacts with Gag but is not incorporated into EIAV particles (83, 112).

EIAV lacks a virus infectivity factor gene, but the *pol* gene of EIAV encodes a dUTPase protein, which is important for replication in non-dividing macrophages (55, 97, 101). Cells of the monocyte/macrophage lineage are the primary targets for EIAV infection. Whereas monocytes are permissive for EIAV infections (89), viral expression occurs only in differentiated macrophages (66). Interestingly, EIAV can utilize a single cellular receptor, designated equine lentivirus receptor-1 (ELR1), to infect target cells (99, 114). Equine lentivirus receptor-1 is a member of the tumour necrosis factor receptor (TNFR) protein family. TNFR-like proteins have also been identified as receptors for certain avian oncoviruses and FIV (CD134) (1, 6, 13, 22, 94, 114).

EIAV is non-pathogenic in humans (88), suggesting that it is an intrinsically safe virus and of interest for therapeutic studies. Therefore, EIAV-based vectors have been used for gene therapy in experimental models of human diseases (3, 4, 35).

FIV

Infection with *Feline immunodeficiency virus* induces an AIDS-like syndrome in cats. The progression of the disease is characterized by typical phases which were initially observed for primate lentiviruses (15). FIV has been currently classified into five subtypes (A-E), based on their *gag* and *env* sequences. The subtypes are distributed according to the geographic location. Besides the domestic cat, many other felids including lions, pumas, and leopards, are infected with species-specific FIVs. Further sequence analyses demonstrated also a species-specific FIV infection in *Hyaenidae* (102). The phylogenetic data indicate that most of these FIV types isolated from different felids are monophyletic (73, 102). However, cross-species transmission of FIV have been observed in wild cats, indicating that repeated and/or multiple historic FIV cross-species felid-to-felid transmission events can occur in the wild or in captivity (16, 31, 71, 73, 102, 105).

FIV shows a tropism for T cells (74), macrophages (14), and cells of the central nervous system (26). In spite of the $CD4^+$ cell tropism, CD4 is not used as binding receptor for FIV. The primary cellular receptor for FIV is feline CD134 (94). Interestingly, FIV can use both feline and human CXCR4 as co-receptor, but cannot use human CD134 (21, 22).

The FIV genomic organization is similar to HIV, nevertheless it has important differences. Like other retroviruses, the integrated provirus is bordered by long terminal repeats and possesses *gag*, *pol*, and *env* genes (FIG. 1). Unlike primate lentiviruses, FIV lacks *nef* (negative regulatory factor), *vpr* (viral protein R), and *vpu* (viral protein U) genes. However, one open reading frame of FIV, termed OrfA, was speculated to be an transactivator like Tat (transactivator of transcription) (23, 96, 107). In addition, OrfA acts similar to Vpr and is also involved in virus release and influences the cell cycle (34). Recently it was shown that OrfA reduces the cell surface expression of the FIV primary binding receptor CD134 (41). Overall, the findings suggest that OrfA may be a multi-functional protein. In contrast to HIV-1 FIV contains a P2 protein (28) instead of P6 protein, which is necessary for virus budding (62). FIV *pol* encodes protease, reverse transcriptase, integrase and also dUTPase. (28). A lack of dUTPase results in significant increase of base changes, primarily G→A transitions, during replication in macrophages (51).

The FIV genome contains a homologous gene to the HIV-1 *vif* gene. Both virus infectivity factors of FIV and HIV-1 act to reduce G→A mutations by preventing cytidine deamination by cellular APOBEC3 (apolipoprotein B mRNA editing enzyme catalytic polypeptide 3) proteins (60, 68, 115).

APOBEC3

The relevance of apolipoprotein B mRNA editing enzyme catalytic polypeptide 3 (APOBEC3) was discovered through the study of Vif. Vif HIV-1 virions produced in permissive cells were able to infect permissive and non-permissive cells, whereas ΔVif virons produced in non-permissive cells were unable to productively infect target cells (33, 80). These findings suggested the existence of an antiviral factor that Vif was able to neutralize (59, 95). This Vif-sensitive cellular factor was described in 2002 by Sheehy *et al.* termed CEM15 (later named APOBEC3G) (92). APOBEC3G was found to be highly expressed in non-permissive cells and poorly expressed or even absent in permissive cells. Meanwhile, it could be shown that other lentiviruses, gammaretroviruses, deltaretroviruses, spumaviruses, LTR/Non-LTR retrotransposons, orthohepadnaviruses, and avihepadnaviruses are also sensitive to APOBEC3 activity (24, 25, 27, 29, 56, 60, 77-79, 81, 87, 103).

APOBEC3 proteins belong to a family of polynucleotide cytidine deaminases that exhibit RNA and DNA editing activity. In humans the APOBEC3 gene family includes seven members (APOBEC3A, -B, -C, -D, -F, -G, -H) (20, 43), whereas the number of APOBEC3 genes varies in other mammals (12, 49, 68, 116). A multiplicity of evolutionary events, including

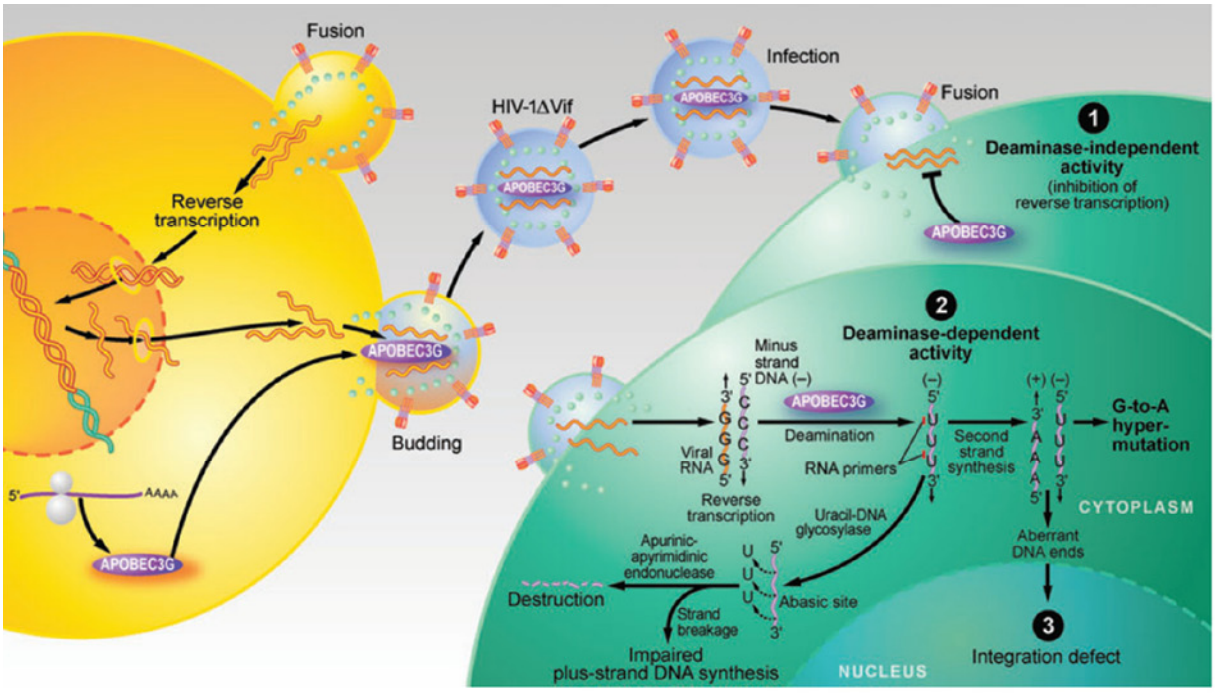

FIG. 3. Model for human APOBEC3G antiviral function. The absence of Vif results in the packaging of APOBEC3G into HIV virions budding from producer cells. In target cells, encapsidated APOBEC3G acts as an inhibitor of virus replication. These inhibitory effects are divided in deaminase-independent and deaminase-dependent activities. Binding of APOBEC3G to HIV-1 RNA prevents the progress of reverse transcriptase and results in a deaminase-independent decrease of early viral reverse transcripts ❶. Single-stranded viral DNA serves as a target for deaminase-dependent activity of APOBEC3G. APOBEC3G causes hypermutation of the minus-strand viral DNA, resulting in the conversion of deoxycytidine to deoxyuridine. Uracil-containing minus-strand DNA can be targeted by uracil DNA glycosylase, which could lead to endonucleolytic cleavage ❷. APOBEC3G may also cause defects in $tRNA^{Lys3}$ primer cleavage leading to the formation of viral DNA with aberrant ends that could interfere with subsequent chromosomal integration ❸. Figure taken from (18).

preservation, deletion, gene duplication, subfunctionalisation, and neofunctionalisation, have led to different numbers of APOBEC3 genes.

All APOBEC3 proteins have one or two conserved cytidine deaminase sites (His-X-Glu-X_{23-28}-Pro-Cys-X_{2-4}-Cys) (43) coordinating Zn^{2+} ions (48). The enzymatic activity results in the hydrolytic deamination at the C4 position of the cytosine base of either RNA or DNA, thereby converting cytidine to uridine.

The best characterized APOBEC3 protein is human APOBEC3G. In absence of Vif_{HIV-1}, APOBEC3G is incorporated into HIV-1 virions through an RNA-dependent interaction with the nucleocapsid (2, 46, 82, 100). Both, nucleocapsid and APOBEC3G are able to bind RNA (8, 54). Incorporated APOBEC3G specifically deaminates cytidine to uridine in newly synthesized single-stranded DNA during reverse transcription, leading to viral genome degradation or hypermutation (10, 37, 50, 60, 64, 115). Uridine-containing viral minus-strand DNA can be targeted by uracil DNA glycosylase, leading to destruction by the apurinic-apyrimidinic endonuclease (85, 111). Non-degraded minus-strand DNA serves as template for plus-strand synthesis, where the deoxyuridine residues promote G→A mutations in the DNA plus-strand. These misincorporations alter open reading frames and introduce inappropriate translation termination codons, and inhibit $tRNA^{Lys3}$-primed reverse transcription, which is essential for chromosomal integration of viral DNA (36, 37, 50, 60, 115) (FIG. 3).

However, APOBEC3 proteins also have deaminase-independent antiviral activities (70). Several studies have shown that deaminase-deficient APOBEC3 mutants are still able to reduce accumulation of reverse transcription products during the early stages of viral replication (9, 40) (FIG. 3). Other studies found that human APOBEC3G is able to inhibit all stages of reverse transcription (42). The ability of APOBEC3 to bind viral RNA appears to be involved in deaminase-independent inhibition (9, 70).

APOBEC and Vif

The interaction of APOBEC3 and Vif proteins is best characterized for human APOBEC3G and Vif_{HIV-1}. The virus infectivity factor forms an interaction between APOBEC3G and an ubiquitin E3 ligase complex consisting of Elongin B and C, Cullin5, and Ring Box-1 (113). This interaction results in polyubiquination, which leads to proteasomal-mediated degradation of the APOBEC3G protein (19, 65, 67, 93, 113) (FIG. 4). For human and African Green Monkey (AGM) APOBEC3G this interaction was shown to be species-specific.

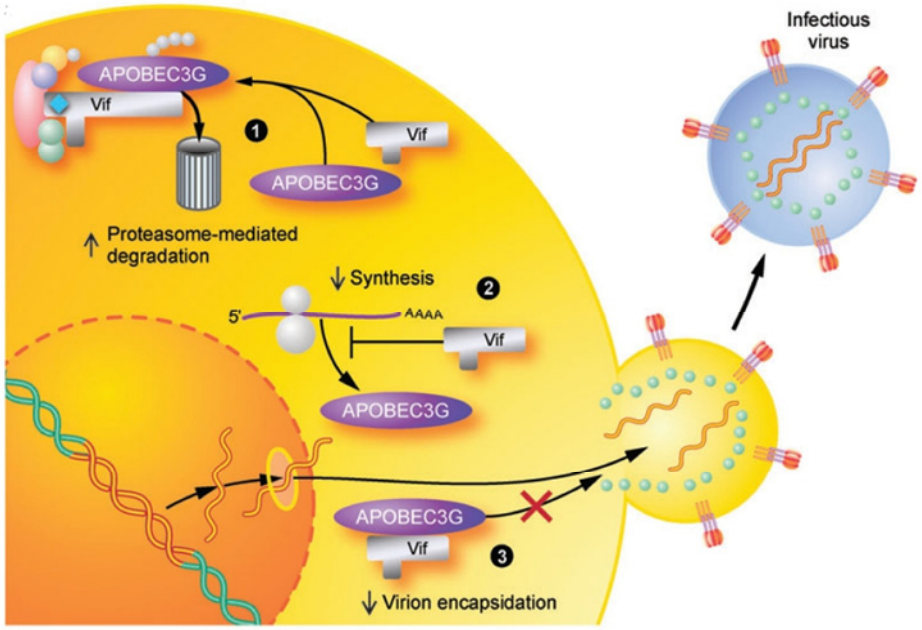

FIG. 4. Model of the interaction of Vif$_{HIV-1}$ and human APOBEC3G. Vif targets the human APOBEC3G protein for proteasomal degradation ❶. The Expression of Vif also partially impairs the translation of human APOBEC3G mRNA ❷. These two effects of Vif effectively eliminate APOBEC3G from the virus-producing cell and lead to production of infectious virions lacking APOBEC3G ❸. Figure taken from (18).

Both APOBEC3G proteins differ in one amino acid which is sufficient for resistance of AGM APOBEC3G against Vif-mediated degradation (11, 61, 84, 110). Equally, AGM but not human APOBEC3G is inactivated by the Vif protein of SIV$_{AGM}$ (11, 84). This species-specific interaction could be an explanation for the host range of the certain primate lentiviruses.

SCOPE OF THIS THESIS

This thesis focuses on the detection and characterization of APOBEC3 proteins of mammalia outside the well-characterized human/primate system. Previous studies have shown an important diversity in quantities, expression levels and antiviral properties of APOBEC3 proteins of different species. For instance, the human genome carries seven APOBEC3 genes, whereas the mouse genome contains only a single APOBEC3 gene. Many details are known about the suppression of productive replication of HIV-1 in non-human cells and the species-specificity of the APOBEC3-Vif interaction. In order to complete these studies *Felis catus* and *Equine caballus* were examined for APOBEC3 genes.

The presence of different feline APOBEC3 proteins leads to the question, whether these proteins show an inhibitory effect against retroviruses and variability in their antiviral activity. Chapter two describes the restriction of HIV-1 in feline cells expressing human CD4- and CCR5-receptors by feline APOBEC3 proteins. This work also investigated the possibility of using *Felis catus* as an animal model for studying HIV-infection / AIDS-progression.

The studies in chapter three extend the analyses of feline APOBEC3 and show the inhibitory activity against feline viruses and SIV, respectively. The parallels between FIV and HIV-1 concerning pathology and molecular structure, were of special interest. In order to show similarities in resistance between FIV and HIV-1, the interaction of feline APOBEC3 and Vif$_{FIV}$ was characterized. The analyses suggest parallels of feline APOBEC3 and human APOBEC3 proteins in their individual specificity and support a complex evolutionary history of expansion, divergence, selection and individual extinction of antiviral APOBEC3 genes.

Another aim of this thesis was to get more details about host cell factor interactions of the more primitive lentivirus EIAV. It was interesting to study the question how EIAV can replicate without a VIF protein in cells that likely express APOBEC3 proteins. In chapter four, equine APOBEC3s were investigated for their antiviral activities and EIAV for its mechanisms of resistance to APOBEC3. Two candidate proteins, S2 and dUTPase, were tested whether they are able to adopt the function of Vif.

Previous studies have suggested the Vif-APOBEC3 interaction to be species-specific and thereby preventing cross-species virus transmission. Chapter five provides a detailed analysis of Vif from FIV and APOBEC3 restriction factors of different felids. The study describes the ability of Vif to counteract APOBEC3 proteins from different felid species, implying that APOBEC3 does not form a block against cross-species transmission between these closely related animals. Finally, Vif of FIV was also found to be expressed in the heterologous HIV-1, strengthening the idea of a cat HIV/AIDS model.

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CHAPTER 2

Multiple Restrictions of Human Immunodeficiency Virus Type 1 in Feline Cells

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The productive replication of human immunodeficiency virus type 1 (HIV-1) occurs exclusively in defined cells of human or chimpanzee origin, explaining why heterologous animal models for HIV replication, pathogenesis, vaccination, and therapy are not available. This lack of an animal model for HIV-1 studies prompted us to examine the susceptibility of feline cells in order to evaluate the cat (*Felis catus*) as an animal model for studying HIV-1. Here, we report that feline cell lines harbor multiple restrictions with respect to HIV-1 replication. The feline CD4 receptor does not permit virus infection. Feline T-cell lines MYA-1 and FeT-1C showed postentry restrictions resulting in low HIV-1 luciferase reporter activity and low expression of viral Gag-Pol proteins when pseudotyped vectors were used. Feline fibroblastic CrFK and KE-R cells, expressing human CD4 and CCR5, were very permissive for viral entry and HIV-long terminal repeat-driven expression but failed to support spreading infection. KE-R cells displayed a profound block with respect to release of HIV-1 particles. In contrast, CrFK cells allowed very efficient particle production; however, the CrFK cellderived HIV-1 particles had low specific infectivity. We subsequently identified feline apolipoprotein B-editing catalytic polypeptide 3 (feAPOBEC3) proteins as active inhibitors of HIV-1 particle infectivity. CrFK cells express at least three different APOBEC3s: APOBEC3C, APOBEC3H, and APOBEC3CH. While the feAPOBEC3C did not significantly inhibit HIV-1, the feAPOBEC3H and feAPOBEC3CH induced G to A hypermutations of the viral cDNA and reduced the infectivity 10- to 40-fold.

Like many retroviruses, human immunodeficiency virus type 1 (HIV-1) has a very limited host range; spreading replication is seen only in Homo sapiens and by artificial inoculation in the close relative the chimpanzee (*Pan troglodytes*) (2, 16), preventing the setup of an efficient small animal model for HIV-1 research. Many reasons argue against the widespread use of chimpanzees in research, including the ethical problems involved in the use of an endangered species, budgetary problems, and the very low induction of simian AIDS either from HIV-1 or its ancestor simian immunodeficiency virus cpz (SIVcpz) in infected chimpanzees (25, 26, 30, 57, 58, 60, 63).

In general, the tropism of HIV-1 in human tissue is determined by the expression of its receptor protein CD4 together with CCR5 or CXCR4 chemokine receptors. Simian CD4 but not murine CD4 supports entry of HIV-1 (15, 33). HIV-1 entry through receptor-mediated membrane fusion is required for reverse transcription of the viral genomic RNA into a double-stranded DNA molecule. Murine T cells show early postentry restriction of HIV-1 at reverse transcription (3). In simian cells, a related restriction of HIV-1, but not of SIVs (5, 10, 53), involves the simian TRIM5α protein, which leads to increased viral uncoating and thereby suppresses reverse transcription (73). Since HIV-1 does not show spreading replication in non-humancells, cell type- and tissue-specific tropism was studied mostly using human cells. Soon after identifying the relevance of the chemokine coreceptors for HIV infection, it was realized that certain receptor- and coreceptor-positive human cells, mostly nondividing, were still resistant to full HIV-1 replication. HIV-1 can infect human cells arrested in the cell cycle at the G0/1a or G2 stage (13, 21, 37). Remarkably, in a few quiescent and nonactivated human cells, such as monocytes and resting peripheral T lymphocytes, HIV-1 is restricted soon after membrane fusion. It has been discussed that a limiting nucleotide pool (31, 83) and/or a unique and un-chracterized activity of low-molecular-weight complexes of a protein called apolipoprotein B-editing catalytic polypeptide 3G (APOBEC3G) (see below) exclusively found in resting cells perturbs reverse transcription in these cells (9). In some experimental systems using nondividing human cells, the nuclear import and/or the integration of viral DNA into chromosomal DNA is also very inefficient (55, 69, 74). This is, however, not a general feature of resting cells in vivo, since human resting T cells residing within lymphoid tissues are permissive for HIV-1 infection (13), indicating that subtle differences in cell physiology play a crucial role in whether a given cell is permissive to HIV-1 replication or not.

Beside these so-called early replication blocks, nondividing human cells also show late blocks (for a review, see reference 81). In dividing murine cells, low levels of transcription by nonfunctional p-TEFb complexes (consisting of cyclin T1 and CDK9) have been observed (7, 17, 32, 79). Murine cells show several additional late blocks of HIV replication, such as disturbed RNA export (47, 75, 85) and processing and assembly

blocks of the viral proteins (6, 45). One of the best-characterized cellular proteins efficiently restricting HIV-1 is the cytidine-deminase APOBEC3G (70). Encapsidation of APOBEC3G and other members of the APOBEC3 family in HIV-1 virus particles leads to deamination of cytosine residues to uracil in growing single-stranded DNA during reverse transcription (8, 24, 34, 42, 43, 84). HIV uses the viral infectivity protein (Vif) to prevent or at least reduce incorporation of APOBEC3G into progeny virions (43, 46, 71). Despite vif expression, low levels of APOBEC3-mediated cytidine deamination are detectable, indicating that even wild-type (wt) HIV-1 is weakly restricted by the presence of APOBEC3 proteins (61). Because the HIV-1 Vif protein exclusively binds and inactivates human APOBEC3 proteins in a species-specific way, HIV-1 is strongly inhibited by the simian and murine orthologues of APOBEC3G (43) that evade HIV-1 Vif counteraction.

Given that there is neither a small animal model nor a primate model available for HIV-1 investigations, rodent systems were developed to model specific steps of HIV-1 infection. Transgenic mice containing full-length or individual genes under the control of non-HIV promoters were used to study the postintegration phase of the viral life cycle. These models provide a means for assessing effects of HIV-encoded proteins in vivo and mammalian responses to different viral gene products (23, 35, 65, 76). In addition, severe combined immunodeficient (SCID) mice, engrafted with human peripheral blood mononuclear cells or fetal thymus and fetal liver cells, have been used as a model for HIV-1 (50, 54). With both SCID models uncontrolled HIV-1 infections, limited to the engrafted cells, peak at 3 to 4 weeks postinoculation and are characterized by variable depletions of the engrafted CD4 T cells and a lack of humoral or cellular responses to the virus (1, 51). To increase the longevity of the engraftment with multilineage hematopoiesis and properties of a more functional human immune system, $Rag2^{-/-}\gamma c^{-/-}$ or NOD/SCID/IL2R$\gamma^{-/-}$ mice engrafted with human hematopoietic stem cells were recently used for HIV-1 infection studies (4, 20, 78).

The potential usefulness of an animal with an intact immune system as a model of HIV-1 infection and disease warrants further efforts directed at an assessment of the limitations and blocks in the viral life cycle in animals that may serve for animal experimentation. The cat (*Felis catus*) is an established animal model for studies of the brain, genetics, pharmacology, nutrition, and virology (59). Unlike rodents, cats are permissive for infection by a lentivirus, the feline immunodeficiency virus (FIV). But FIV is only distantly related to HIV-1; thus, studies of FIV are suitable only to a limited extent for understanding HIV-1 pathogenesis. Initial observations of the host range of HIV-1 in feline cells showed a lack of reverse transcriptase (RT) production after virus inoculation (36), but subsequent experiments using vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV vectors containing cytomegalovirus (CMV) promoters demonstrated robust gene transfer into feline cell lines (28, 64, 66). Cellular factors restricting HIV-1 in the cat are currently unknown. However, in a recent study of feline foamy viruses (FFV), we characterized a feline APOBEC3 protein (39), demonstrating that in addition to primates and rodents, felines also express cytosine-deaminases restricting retroviral replication. Because feline cells are known to be highly permissive for reporter gene expression by HIV vectors, we were curious to extend this finding and to explore the use of the cat as a potential small-laboratory animal model for HIV-1 infection.

We show here that cell lines derived from Felis catus show several restrictions to HIV-1 replication regarding entry, particle release, and particle infectivity. The feline APOBEC proteins 3H and 3CH were identified as potent inhibitors of HIV-1 in feline cells.

MATERIALS AND METHODS

Cells and transfections. The adherent human cell lines HOS (American Type Culture Collection [ATCC] CRL-1543), HOS.CXCR4 (National Institute for Biological Standards and Control [NIBSC] ARP5000), HOS.CCR5 (NIBSC ARP5001), HOS.CD4.CCR5 (NIBSC ARP078), HT1080 (ATCC CCL121), and 293T and feline cell lines CrFK (ATCC CCL-94; feline kidney cells) and KE-R (feline embryonic fibroblast cells; a gift of Roland Riebe, Friedrich-Loeffler Institut, Riems, Germany) were maintained in Dulbecco's high-glucose modified Eagle's medium (Dulbecco's modified Eagle's medium complete; Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.29 mg/ml L-glutamine, and 100 units/ml penicillin/streptomycin. Cells of the human T-cell line A3.01 (NIBSC ARP098) were cultured in complete RPMI 1640 medium–10% heat-inactivated FBS–0.29 mg/ml L-glutamine–100 units/ml penicillin/streptomycin. Cells of the feline T-cell lines MYA-1 (ATCC CRL-2417) and FeT-1C (ATCC CRL-11968) were cultured in complete RPMI 1640–0.29 mg/ml L-glutamine–10 mM HEPES–1.0 mM sodium pyruvate supplemented with 0.05 mM 2-mercaptoethanol, 100 units/ml human recombinant interleukin-2 and 10% heat-inactivated FBS, and 100 units/ml penicillin/streptomycin. Plasmid transfection into 293T and CrFK cells was done with Lipofectamine 2000 according to the instructions of the manufacturer (Invitrogen). Feline CD4 was amplified from cDNA of feline peripheral blood mononuclear cells after activation with phytohemagglutinin (3 μg/ml), the forward primer 5'fCD4-EcoRI(5'-GAATT CATGAATCAAGGAGCCGTTTTTAGG-3'), the reverse primer 3'fCD4-BglII (5'-AGATCTTCAAATGGGATTACATGTCTTCTG-3'), and Pwo polymerase (Roche Diagnostics). Thirty cycles were run at 94°C for 30 s, 58°C for 1 min, and 72°C for 2 min. PCR products were cloned into pMSCVneo (Clontech) by use of EcoRI and BglII restriction sites. The identity of the resulting pMSCVneo-fCD4 was confirmed by sequencing. pMSCVneo-hCD4 was generated by transferring hCD4 cDNA from T4-pMV7 (41) into pMSCVneo by use of EcoRI restriction sites. Retroviral vector stocks of pBABE-CCR5 (puro) (12), pMSCVneo-hCD4, and pMSCVneo-fCD4 were used to infect cells. After selection with puromycin for CCR5 and G418 for CD4 vectors, receptor expression was confirmed by flow cytometry. The cells were stained for human CD4 with SK3-PerCP (BD PharMingen), for feline CD4 with 3-4F4-PE (SouthernBiotech), for human CCR5 with 2D7-PE (BD PharMingen), and for human CXCR4 with 12G5-PE (BD PharMingen).

Viruses and infections. Replication-competent HIV-$1_{NL4.3}$ and HIV-$1_{NL\text{-}BaL}$ (NL4.3 with the env BaL) (44) virus stocks were prepared by harvesting the supernatant of transfected 293T cells. To generate VSV-G-pseudotyped HIV-1, 293T cells were cotransfected with HIV-1 DNA (pNL4.3; pNL-BaL) and pMD.G VSV-G expression plasmid (14). HIV-1 single-cycle luciferase reporter viruses (HIV-Luc) were produced by cotransfecting 293T cells with pNL-LucRE (a gift from Nathaniel R. Landau; 45), JR.FL (pcJR.FL; 12), L102, a variant of the C-terminally truncated (at amino acid 712) HIV-1 Env strain BH10 (pcL102), HXB2 (pSV7d; 62), BaL.01 (HIV-1 clone BaL.01; 38), BaL.26 (HIV-1 clone BaL.26; 38), or VSV-G expression vector. Δ*vif* HIV-Luc was produced by cotransfecting 293T cells with pNL-LucR$^-$E$^-$Δ*vif* (43) and VSV-G expression plasmid. Single-cycle HIV-1-green fluorescent protein (GFP) (VSV-G) expressing enhanced GFP (EGFP) by an internal CMV promoter were generated by transfection of 293T with pHIV.NL4-3ΔE-EGFP (52) together with pMD.G. EGFP vectors were titrated by serial dilutions using HT1080 cells as described previously (52), 4 x 10^5 cells were transduced using a multiplicity of infection (MOI) of 5, and GFP expression was determined by flow cytometry 3 days posttransduction. FIV single-cycle luciferase (FIV-Luc) vectors were produced by cotransfecting 293T cells with pFP93 (a gift of Eric M. Poeschla; 40), pvLucFIVΔ*env*, and pMD.G. pvLucFIVΔ*env* vector was derived from p34TF10, a replication-competent molecular clone of FIV, contains a 2 kb internal deletion in env, and has most of *gag-pol* region replaced by the firefly luciferase gene under the control of an internal CMV promoter. RT of viruses was determined by use of a Cavidi HS Lenti RT kit (Cavidi Tech). Alternatively, viruses were quantified using an HIV-1 p24 antigen enzyme-linked immunosorbent assay

(ELISA) based on a previously published method (48, 49). Briefly, p24 antigen is captured from a detergent lysate of virions (1% Empigen; Calbiochem) by use of a polyclonal antibody (D7320 sheep anti-HIV-1-p24 gag; Aalto Bio Reagents, Dublin, Ireland) adsorbed to a solid phase. Bound p24 is detected using an alkaline phosphatase-conjugated anti-p24 monoclonal antibody (BC 1071-AP alkaline phosphatase conjugate of anti-HIV-1-p24 mouse monoclonal antibody; Aalto Bio Reagents, Dublin, Ireland) and a luminescence detection system (Tropix ELISA-Light immunoassay system; Applied Biosystems) and analyzed using a Berthold MicroLumat Plus luminometer. For reporter virus infections, the adherent cells were seeded at 2.0 x 10^3 cells/well per day before transduction and suspension cells were seeded at 5.0 x 10^4 cells/well on the day of transduction in 96-well plates and then infected with reporter virus stocks normalized for RT. Firefly luciferase activity was measured according to the manufacturer's directions 3 days later with a Steadylite HTS reporter gene assay system (PerkinElmer) on a Berthold MicroLumat Plus luminometer.

APOBEC3 expression and plasmids. Feline APOBEC3C (previously termed feAPOBEC3 [fe3]; GenBank accession no. AY971954) was described previously (39). Feline APOBEC3H and feline APOBEC3CH cDNAs were identified by using 5‘ and 3‘ rapid amplification of cDNA ends (RACE) reactions (5‘/3‘-RACE kit; Roche Diagnostics) employing total RNA from CrFK cells. For full-length expression cloning of C-terminal hemagglutinin (HA)-tagged feline APOBEC3H, forward primer fAPO-29 (5‘-TGCATCGGTACCTGGAG GCAGCCTGGGAGGTG-3‘) and reverse primer fAPO-28 (5‘-AGCTCGA GTCAAGCGTAATCTGGAACATCGTATGGATATTCAAGTTTCAAAT TTCTGAAG-3‘) and Pwo polymerase (Roche Diagnostics) were used; for feline APOBEC3CH, forward primer fAPO-30 (5‘-TGCATCGGTACCACC AAGGCTGGAGAGAGGAATGG-3‘) and reverse primer fAPO-28 and Pwo polymerase were used. Each of 30 cycles was run at 94°C for 30 s, 58°C for 1 min, and 72°C for 2 min, PCR products were cloned into the KpnI and XhoI sites of pcDNA3.1(+), and correct clones were identified by sequencing. Expression studies of feline APOBEC3 RNA of CrFK cells were done by RT-PCR using total RNA, Taq polymerase (QIAGEN), and forward primer fAPO3F-18 (5‘-TAGAAGCTTACCAAGGCTGGCGAGAGGAATGG-3‘) and reverse primer fAPO3F-19 (5‘-AGCTCGAGTCAAGCGTAATCTGGA ACATCGTATGGATACCTAAGGATTTCTTGAAGCTCTGC-3‘) for feline APOBEC3C, forward primer fAPO3F-9 and reverse primer fAPO-26 (5‘-CTGCCCGAAGGCACCCTAATTC-3‘) for feline APOBEC3H, and forward primer fAPO3F-11 (5‘-ACCAAGGCTGGCGAGAGGAATGG-3‘) and reverse primer fAPO-27 (5‘-TCGTACTCGAGGCAGTTTATGAAGCATT GAGATGC-3‘) for feline APOBEC3CH. PCRs were run for 30 cycles of 94°C for 30 s, annealing for 1 min for feA3C at 62°C, for feA3H at 61°C, and for feA3CH at 60°C, and 72°C for 2 min.

Immunoblot analysis. Cells were infted with HIV-1NL.BaL(VSV-G) or co-transfected with plasmids for HIV-Luc or Δ*vif*HIV-Luc, and APOBEC3-HA expression plasmids and lysates and virions were prepared 3 days later. Virions were pelleted by centrifugation of filtered culture supernatant through a 20% sucrose cushion at 35,000 rpm in an SW40Ti rotor for 1.5 h and lysed in lysis buffer (100 mM NaCl, 10 mM EDTA, 20 mM Tris [pH 7.5], 1% Triton X-100, 1% sodium deoxycholate). Cell lysates were prepared by removing infected cells from the medium, washing the cells with phosphate-buffered saline, and lysing the cells in lysis buffer. Protein in the lysates was quantitated using Coomassie blue reagent (Bio-Rad). Lysates containing 20 g of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride filters. Filters were probed with mouse anti-capsid p24 hybridoma supernatant (α-p24 183-H12-5C; provided by Egbert Flory) (1:50 dilution) or HIV-1 Vif antiserum (HIV-1HXB2 Vif antiserum; 19) (1:2,000 dilution) or anti-HA antibody (MMS-101P; Covance) (1:6,000 dilution) or mouse anti-α-tubulin (clone B5-1-2; Sigma-Aldrich) (1:4,000 dilution) followed by horseradish peroxide-conjugated rabbit anti-mouse antibody (/-mouse-IgG-HRP; Amersham Biosciences) and developed with ECL chemiluminescence reagents (Amersham Biosciences).

Sequencing of viral reverse transcripts. HOS cells (1 x 10^6) were infected with DNaseI (Roche)-treated HIV-Luc(VSV-G) (1,000 pg RT). At 10 h postinfection, cells were washed with phosphate-buffered saline and DNA was isolated using a DNeasy DNA isolation kit (QIAGEN). A 600 bp fragment covering long terminal repeat gag (LTR-gag) was amplified using Taq DNA polymerase (QIAGEN) and the primers MH 531 (5‘-TGTGTGCCCGTCTGTTGTGT-3‘) and CM100 (5‘-TGGAGGTTCTGCACTATAGGG-3‘). Each of thirty cycles was run at 94°C for 30 s, 58°C for 1 min, and 72°C for 2 min, and PCR products were cloned into TOPO TA-cloning pCR4 vector (Invitrogen) and sequenced. The nucleotide sequences of at least 10 independent clones were analyzed.

Nucleotide sequence accession numbers. The sequences reported in this paper have been deposited in the GenBank database as follows: feline APOBEC3H (accession no. EF173020) and feline APOBEC3CH (accession no. EF173021).

RESULTS

HIV-1(VSV-G) infects feline adherent cells but is restricted in feline T cells. To evaluate the cat (*Felis catus*) as an animal model for HIV-1, established feline cell lines were used to characterize HIV-1 replication. Because Tat transactivation of the HIV-LTR in murine cells is inefficient (7, 17, 32, 79), we were interested in discovering whether HIV-LTR-driven reporter viruses in cat cells would generate high expression of the reporter protein. Feline adherent cell lines (KE-R and CrFK) and feline T-cell lines (MYA-1 and FeT-1C) were transduced with VSV-G-pseudotyped HIV-1 luciferase reporter viruses. To draw a comparison to results obtained in investigations of the feline cells, the permissive human cell lines HOS and A3.01 were coinoculated with the luciferase virus. Pseudotyping of the particles with VSV-G allowed infection of cells that were negative with respect to HIV receptor expression. The virus particles were produced by transient transfection in human 293T cells, and 2, 20, and 200 pg RT were used for transduction. At 3 days postinfection (dpi) luciferase activity in feline and human cells was analyzed. The results presented in Fig. 1A (left panel) show that CrFK cells had equal luciferase activity levels and that KE-R cells had two- to fourfold-lower luciferase counts than HOS cells. In contrast, the feline T-cell lines MYA-1 and FeT-1C showed 5- to 12-fold-lower luciferase activity levels than the human T-cell line A3.01. The results of these experiments indicate that the degree to which feline fibroblasts were permissive for HIV-1 transduction was similar to that seen with the human HOS cells, suggesting efficient LTR-driven transcription.

To confirm that the feline T-cell lines used are susceptible to retrovirus transduction and luciferase reporter gene expression, a different lentiviral system, VSV-G-pseudotyped FIVLuc was analyzed. The single-round replicating vector uses an internal CMV promoter for luciferase expression. At 3 days posttransduction we detected equal levels of luciferase activity in the human A3.01 and feline CrFK cells (Fig. 1A, center panel). FIV-Luc expression was only slightly (1.3- to 1.7-fold) higher in the human A3.01 cells than in the feline Fet-1C and MYA-1 cells. To rule out reduced HIV-LTR-driven expression in the feline T cells, we transduced the cells with a single-round HIV-1 vector which uses an internal CMV promoter to express the GFP gene (HIV-GFP). The HIV-GFP vector particles were also VSV-G pseudotyped. We inoculated the cells with at an MOI of 5 and analyzed the GFP expression by flow cytometry at 3 days posttransduction. The results obtained with the HIV-GFP vector resembled the previous results seen using the HIV-Luc transductions (Fig. 1B): 99% of A3.01 cells were positive for GFP expression, with a mean fluorescence intensity of 2,080, while in the culture of feline MYA-1 cells only 11% green cells were present, with a fivefold-lower mean fluorescence intensity of 414. We detected also a low mean GFP fluorescence intensity of 113, with 26% green cells, in the analyzed feline FeT-1C cells. Analogous results were obtained using a CMV promoter with HIV-2–GFP or SIVPBj-GFP vector (data not shown). Taken together, the data indicate that CMV promoter-driven expression of a reporter gene works in a FIV vector with similar levels of efficiency in human and feline T-cell lines. Apparently, HIV-1 vectors expressing GFP by use of an internal CMV promoter do not overcome the low level of

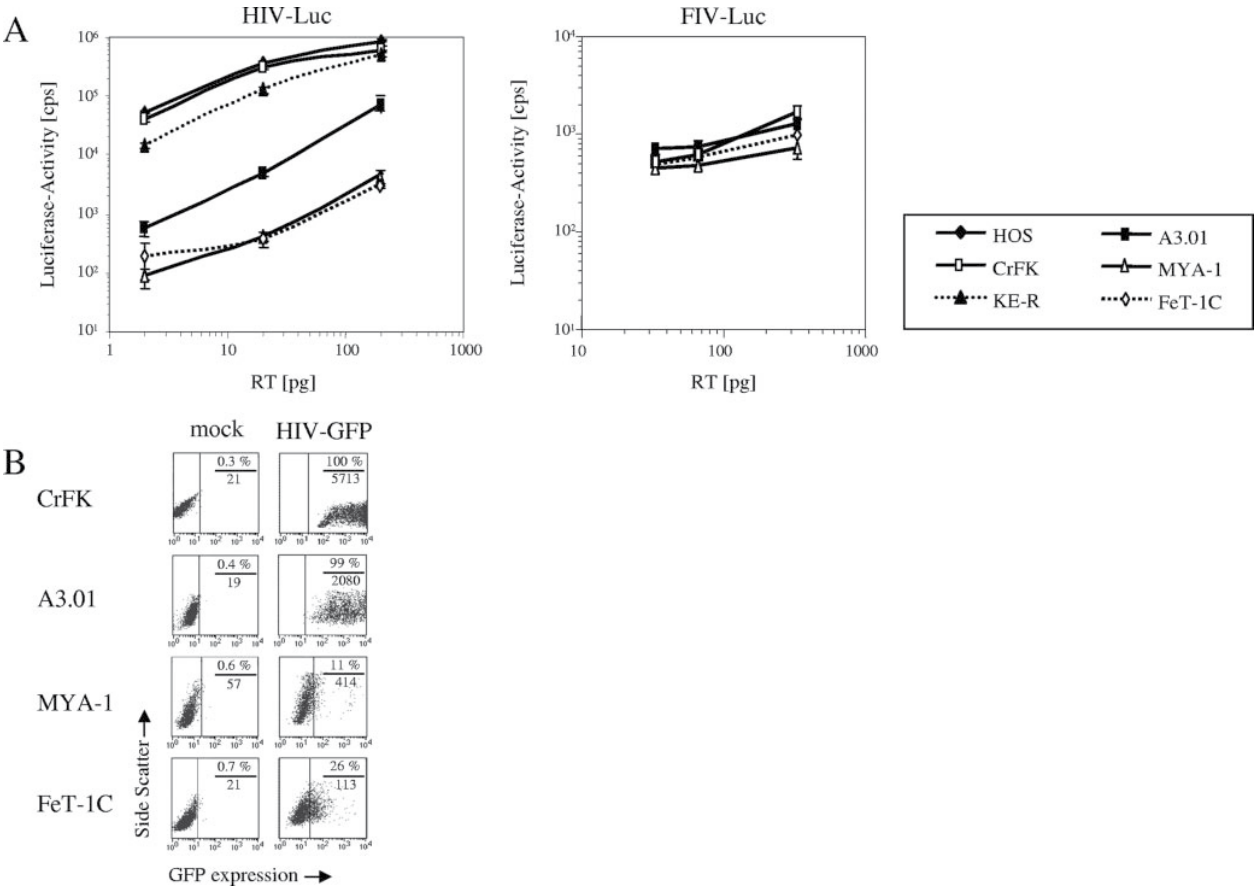
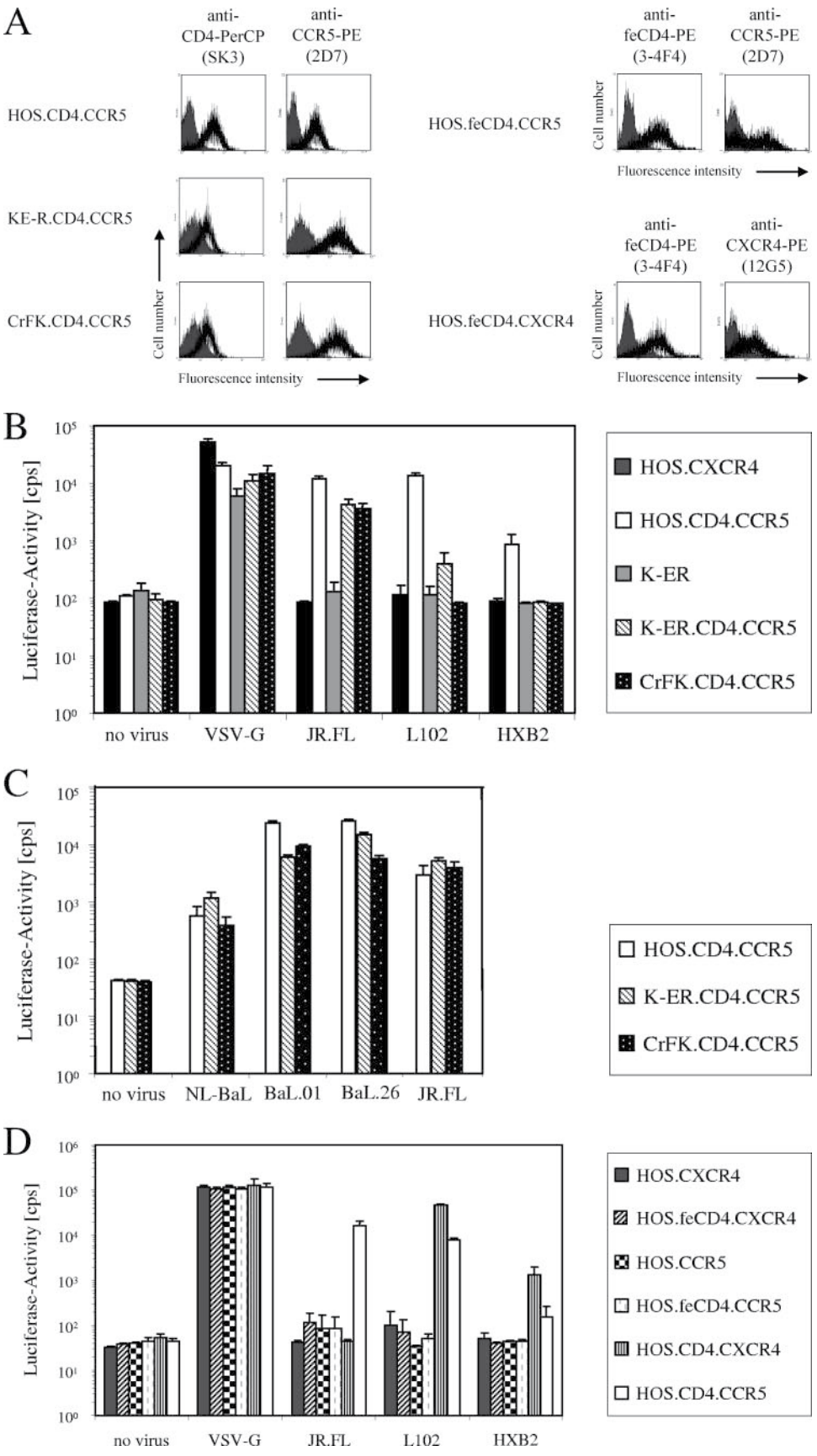


FIG. 1. HIV-1 infects feline adherent cells but is restricted in feline T cells. (A) Left panel: adherent cell lines (human [HOS] and feline [CrFK, KE-R]) and T-cell lines (human [A3.01] and feline [MYA-1, FeT-1C]) were infected with increasing amounts of HIV-Luc(VSV-G). Luciferase activity (in counts per second) was determined at 3 dpi. Center panel: CrFK, A3.01, MYA-1, and FeT-1C cells were inoculated with increasing amounts of FIV-Luc(VSV-G). Luciferase activity was determined at 3 dpi. (B) CrFK, A3.01, MYA-1, and FeT-1C cells were transduced using HIV-GFP(VSV-G) at an MOI of 5. GFP fluorescence was determined 3 dpi by flow cytometry. The data are presented as side scatter versus the intensity of green. The quantity (percent) of GFP-positive cells over the threshold is indicated above of the horizontal line in each panel; mean fluorescence intensity is specified below the horizontal line.

expression of HIV in feline T-cell lines. The data also suggest that the HIV restriction in the feline T-cell lines is not related to cytoplasmic entry of the viral particles by VSV-G.

Feline cells expressing human CD4/CCR5 are permissive for HIV-1 reporter virus transduction. Because the feline CrFK and KE-R cells were more permissive for HIV-1 infection than the feline T-cell lines, we stably transduced CrFK and KE-R cells with retroviral vectors expressing human CD4 (huCD4) and huCCR5. Flow cytometry was used to confirm cell surface expression of huCD4 and huCCR5 in the CrFK.CD4.CCR5 and KE-R.CD4.CCR5 cells. The results demonstrated levels of expression of CD4 and CCR5 similar (CD4 with lower and CCR5 with higher expression) to those seen with the cell line HOS.CD4.CCR5 used as a reference (Fig. 2A). In order to find out whether the human receptors provide entry for HIV-1 into feline cells, we generated HIV-Luc reporter viruses pseudotyped with HIV-1 Env surface proteins. We used expression plasmids for CCR5-tropic JR.FL env and for env genes of HIV-1 strains L102 and HXB2 using CXCR4 as coreceptor. After transduction with JR.FL-pseudotyped reporter virus, feline CrFK.CD4.CCR5 and KE-R.CD4.CCR5 cells exhibited reporter activity as high as that found in human HOS.CD4.CCR5 cells (Fig. 2B and C). Because we planned to use replication-competent CCR5-tropic HIV-1 (NL-BaL; 44) for spreading-replication studies, we also pseudotyped HIV-Luc with BaL Env (Fig. 2C). Since there was no BaL *env* expression plasmid encoding the BaL gene of pNL-BaL available, we used BaL.01 and BaL.26; in addition, we cotransfected HIV-Luc with NL-BaL. The BaL gene in HIV-$1_{NL\text{-}BaL}$ differs from BaL.01 by 29 and from BaL.26 by 37 amino acid positions in gp160 (38). All BaL-pseudotyped HIV-Luc particles transduced the HOS.CD4.CCR5, CrFK.CD4.CCR5, and KE-R.CD4.CCR5 cells with similar levels of efficiency (Fig. 2C). Cells inoculated with the mixture of HIV-Luc/NL-Bal showed 10-


A
anti-CD4-PerCP (SK3)
anti-CCR5-PE (2D7)
HOS.CD4.CCR5
KE-R.CD4.CCR5
CrFK.CD4.CCR5
Cell number
Fluorescence intensity
anti-feCD4-PE (3-4F4)
anti-CCR5-PE (2D7)
HOS.feCD4.CCR5
anti-CXCR4-PE (12G5)
HOS.feCD4.CXCR4
B
Luciferase-Activity [cps]
no virus
VSV-G
JR.FL
L102
HXB2
HOS.CXCR4
HOS.CD4.CCR5
K-ER
K-ER.CD4.CCR5
CrFK.CD4.CCR5
C
NL-BaL
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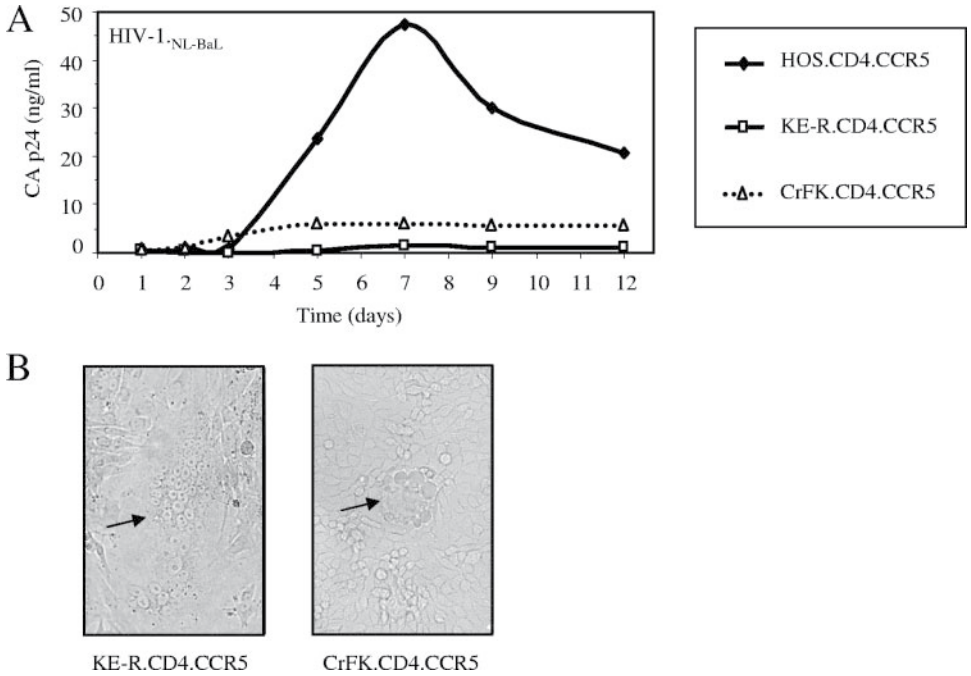

FIG. 3. Feline cells expressing HIV receptors do not support spreading replication of HIV-1. (A) HOS.CD4.CCR5, KE-R.CD4. CCR5, and CrFK.CD4.CCR5 cells were infected with R5-tropic HIV-$1_{NL\text{-}Bal}$ at an MOI of 0.05. Supernatant virions were quantified by p24 ELISA at the indicated days. (B) HIV-$1_{NL\text{-}Bal}$-infected feline cells, KE-R.CD4.CCR5 cells, and CrFK.CD4.CCR5 cells showed syncytium formation (arrows) at 3 to 4 dpi (magnification, x10).

fold-lower luciferase activity, most likely because the replication-competent virus induced massive syncytium formation at 2 to 3 dpi. The X4-tropic envelopes (L102, HXB2) did not allow infection of the huCD4-positive feline cell lines, while reporter viruses carrying either of these glycoproteins infected the CD4-expressing HOS.CD4.CCR5 cells. These results could imply that endogenously expressed CXCR4 protein in HOS cells, but not in feline cells, efficiently supports HIV-1 infection. The cell lines expressing no CD4 (HOS.CXCR4 and K-ER) showed luciferase activity only after inoculation with VSV-G-pseudotyped HIV-Luc particles. In experiments using human HOS cells expressing the feline CD4 protein together with huCCR5 or huCXCR4 (HOS.feCD4.CXCR4, HOS.feCD4. CCR5) (Fig. 2A), luciferase activity of HIV-Luc (JR.FL or L102 or HXB2) was not detectable (Fig. 2D). These results argue against the use of feline CD4 as a potential HIV-1 entry factor.

Lack of spreading replication of HIV-1 in feline cells expressing human entry receptors. In order to further evaluate the capacity of the feline CrFK.CD4.CCR5 and KE-R.CD4.CCR5 cells to support HIV-1 replication, they were infected at a low MOI (0.05) with replication-competent HIV-$1_{NL\text{-}BaL}$ (44). Quantification of released CA.p24 in the supernatant of infected cells by ELISA showed a peak of viral replication in the human HOS.CD4.CCR5 cells at 7 dpi (Fig. 3A). Supernatants of infected feline cells yielded only background levels of p24, indicating the absence of spreading replication in the feline fibroblasts. Interestingly, the HIV-inoculated feline cells showed syncytium formation at 3 to 4 dpi (Fig. 3B), suggesting successful initial infection of some cells with HIV-$1_{NL\text{-}BaL}$.

Processing and release of HIV-1 proteins in feline cells. Since the feline CrFK.CD4.CC5 and KE-R.CD4.CCR5 cells were permissive with respect to transduction by HIV-Luc reportervirus whereas cultres infected with the replication-competent HIV-$1_{NL\text{-}Bal}$ did not show viral spreading, we wanted to investigate the molecular basis of this restriction. In a first step we characterized the expression patterns of viral proteins in feline and human cell lines. To ensure a single round of infection, receptor-negative feline cell lines (KE-R,

FIG. 2. Feline cells expressing human CD4/CCR5 are permissive for HIV-1 reporter virus transduction. (A) Receptor expression of human CD4, feline CD4 (feCD4), human CCR5, and human CXCR4 on HOS, KE-R, and CrFK cells stably transduced by retroviral expression vectors (black) and mock transduced (grey). The cells were stained for human CD4 with SK3-PerCP, feline CD4 with 3-4F4-PE, human CCR5 with 2D7-PE, and human CXCR4 with 12G5-PE. Fluorescence intensity was analyzed by flow cytometry. (B and C) Human and feline cell lines coexpressing human CCR5 and human CD4 were infected with HIV-Luc pseudotyped with VSV-G- or HIV-1-derived envelopes (JR.FL, BaL.01, BaL.26, L102, HXB2). NL-BaL, virus generated by cotransfection of HIV-Luc and HIV-$1_{NL\text{-}BaL}$. Luciferase activity (in counts per second) was determined 3 dpi. Cells expressing no CD4 (HOS.CXCR4 and K-ER) were used as controls. (D) Human HOS cells expressing feline CD4 (feCD4) or human CD4 (CD4) together with human CCR5 or human CXCR4 were infected with HIV-Luc pseudotyped with VSV-G- or HIV-1-derived envelopes (JR.FL, L102, HXB2). Luciferase activity (in counts per second) was determined 3 dpi. Cells expressing no CD4 (HOS.CXCR4 and HOS.CCR5) were used as controls.

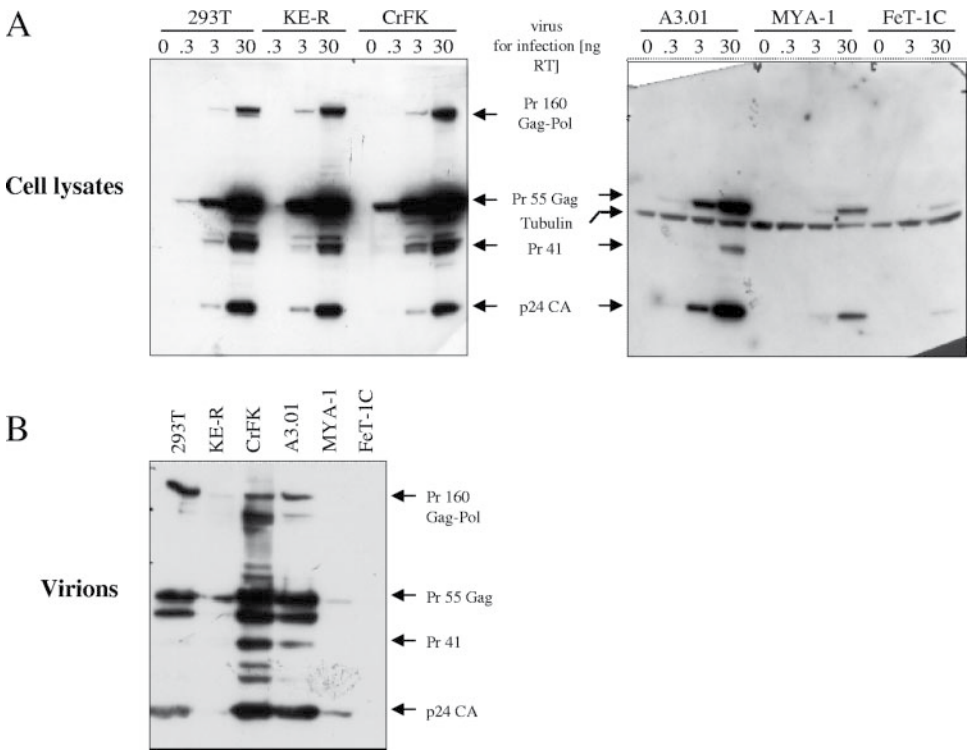

FIG. 4. Reduced HIV-1 Gag expression in feline T cells and restricted HIV-1 release from feline KE-R cells. The results of immunoblot analysis of HIV-1 Gag protein expression and processing by feline and human cells are shown. 293T, KE-R, CrFK, A3.01, MYA-1, and FeT-1C cells were infected with the indicated amounts of HIV-$1_{NL\text{-}Bal}$(VSV-G). At 3 dpi, lysates of the cells were prepared (A) and virions pelleted from the supernatants were analyzed on immunoblots using p24-specific monoclonal antibody (B). For cell lysates of T cells, immunoblots were coprobed using anti-tubulin antibody to show equal sample concentrations. For virions, equal volumes corresponding to 0.5 ml supernatant of cells infected with 30 ng RT were loaded per lane. Gag precursor proteins (Pr 160 Gag-Pol, Pr 55 Gag, Pr 41) and p24 capsid protein (p24 CA) are indicated by arrows.

CrFK, MYA-1, FeT-1C) and human cell lines (293T, A3.01) were used for infection with VSV-G-pseudotyped HIV-1 [NL-BaL(VSV-G)] and analyzed. We used 0.3, 3.0, and 30.0 ng RT for infection, carefully washed the cells to remove the input viral particles at 5 h postinfection, and studied the expression of the HIV-1 Gag/Gag-Pol proteins by anticapsid immunoblotting at 3 dpi. Cell lysates of the infected 293T, KE-R, and CrFK cells showed comparable high levels of expression of the HIV-1 proteins that were dose dependent (Fig. 4A). A significant but lower level of expression was detectable in the A3.01 cell line. Cell lysates of the feline T-cell lines MYA-1 and FeT-1C showed only weak signals with even the highest amount of input virus. The processing patterns in human and feline cells showed no obvious differences. In addition, viral particles released from cultures initially inoculated with 30 ng RT were collected and purified by centrifugation through a sucrose cushion and regular aliquots were analyzed by immunoblotting (Fig. 4B). While the two human cell lines (293T and A3.01) released similar amounts of HIV-1 particles, the feline CrFK cells showed two- to threefold more particles in the cell culture supernatant. In stark contrast, in repeated experiments the feline KE-R, MYA-1, and FeT-1C cells showed no or almost no particles released into the supernatants. This finding correlates with the modest amounts of intracellular HIV Gag in MYA-1 and FeT-1C cells, whereas KE-R cells clearly showed a substantial block in particle release.

HIV-1 virions produced in CrFK cells show low infectivity. The lack of virus particle release in KE-R cells is probably a major reason for the replication block of HIV-1 in KE-R.CD4.CCR5 cells (Fig. 3A). Additional restrictions in feline cells must exist, since CrFK.CD4.CCR5 cells also did not allow spreading HIV-1 replication, although efficient release of particles occurred in infected CrFK cells. In order to study the infectivity of virus particles, we generated HIV-Luc(VSV-G) reporter virions in feline CrFK and human 293T cells by plasmid transfection. The collected viral vectors were normalized by RT activity and used to infect human HOS and feline CrFK cells. HIV-Luc(VSV-G) produced from 293T cells showed high luciferase activity. In contrast, particles from CrFK cells showed only background luciferase activity in human and in feline cells (Fig. 5A), suggesting that these particles had very low infectivity. Several attempts to produce HIV-Luc(VSV-G) in K-ER cells by plasmid transfection were unsuccessful (data not shown).

To identify the cause of the reduced infectivity of CrFK-derived HIV-1 particles, we treated the virus-containing supernatant with DNase I to remove potentially contaminating plasmid DNA and infected human HOS cells. Reverse transcription products were amplified using PCR primers specific for late-RT products (a 600 bp fragment covering LTR-gag) at 10 h postinfection, cloned, and analyzed by sequencing (Fig. 5B). HIV genomes derived from human 293T cells showed no

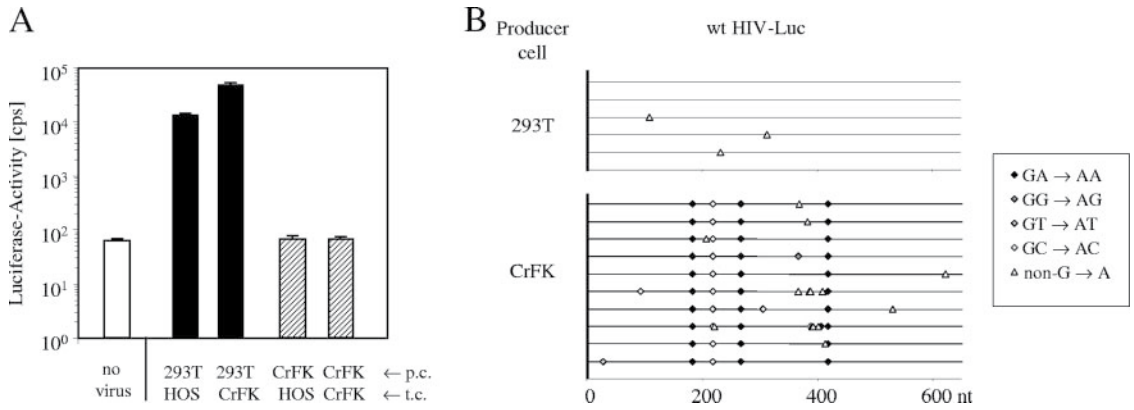

FIG. 5. HIV-1 particles produced in feline CrFK cells show low infectivity. (A) HIV-Luc(VSV-G) was produced in human 293T and feline CrFK cells (producer cells [p.c.]) by plasmid transfection. Normalized viruses containing supernatants were used to infect human HOS and feline CrFK cells (target cells [t.c.]). Luciferase activity (in counts per second) was determined 3 dpi. no virus, uninfected cells. (B) Genome editing of HIV-Luc(VSV-G) produced in 293T or CrFK cells. A fragment in LTR-gag was amplified from reverse transcripts 10 h postinfection. At least 5 to 10 independent nucleotide sequences were determined. The mutations in the clones of each group are shown. Each mutation is indicated and coded with respect to the nucleotide substitution.

G→A exchanges, but G→A substitutions were highly enriched (0.72%) in reverse transcripts of HIV particles made in feline CrFK cells (Table 1). The number of G→A exchanges ranged between four and seven per sequence (Fig. 5B). When we analyzed the positive strand for the sequence context which contained the G3A hypermutations, 25.5% were GG to AG changes, 68% were GA to AA exchanges, and only 6.5% were GC to AC or GT to AT mutations (Fig. 6D). Mutations of a guanosine-purine sequence to an adenosine-purine sequence on the DNA positive strand are typically found in APOBEC3-edited retroviral genomes (8, 24, 34, 39, 42, 43). In summary, the mutation rates showed a strong correlation with the infectivity levels of the HIV particles made in 293T and CrFK cells, explaining the lack of virus spread in CrFK.CD4.CCR5 cells.

Feline CrFK cells express several APOBEC3 RNAs. It has been shown that wt HIV-1 can replicate in the presence of human APOBEC3G; however, its infectivity is dramatically reduced by the presence of heterologous, nonhuman APOBEC3 proteins (43). Based on this observation and the presence of typical G→A mutations in cDNAs from CrFK-derived particles, we assumed that feline APOBEC3 deaminases are a major reason for the low infectivity of HIV-1 produced in CrFK cells. Recently, we described feAPOBEC3C (feA3C; previously termed feAPOBEC3 [fe3]) (39) as being expressed in CrFK cells. For the present study we cloned two additional novel feline cDNAs, termed feAPOBEC3H and feAPOBEC3CH (feA3H and feA3CH), from CrFK-derived RNA by a combination of RT-PCR and 5‘- and 3‘-RACE techniques. The feA3CH cDNA is composed of the fused open reading frames of feA3C and feA3H.We propose a nomenclature classification based on the overall consensus and positions identical between feline and human APOBEC3s: feline APOBEC3C has 43.8% amino acid identity to human APOBEC3C, and feline APOBEC3H shares 44.4% identical amino acids with human APOBEC3H. Using diagnostic PCR primers we confirmed expression of the feA3s in CrFK cells (Fig. 6A). The sizes of the detected amplification products are in line with the structure of the cDNAs. In several experiments using different primer and PCR protocols for amplification of the three feA3 cDNAs, we always observed significantly more PCR product for feA3C than for feA3H and feA3CH (Fig. 6A and data not shown). We used the cloned feA3H and feA3CH cDNAs to generate HA-tagged expression plasmids.

Feline APOBEC3 proteins inhibit the infectivity of HIV-1. To assess the antiviral activity of feA3 proteins, HIV-Luc reporter viruses were generated in human 293T cells in the

TABLE 1. Sequence characteristics of HIV-Luc DNA genomes of virions derived from CrFK cells and 293T-expressing feline APOBEC3 proteins (feA3C, feA3H, feA3CH) or empty expression plasmid

Producer cell and protein or plasmid (vector)	No. of sequences analyzed	No. of G→A mutations/no. of other mutations	No. of mutations per 100 nucleotides	No. of clones without G→A editing	Minimal no. of G→A per clone	Maximal no. of G→A per clone	Average no. of G→A per clone	No. of G→A exchanges per 100 Gs	No. of G→A exchanges per 100 nucleotides
CrFK									
Vector	10	47/14	0.94	0	4	7	4.7	2.7	0.72
293T									
Vector	10	0/7	0.11	10	0	0	0	0	0
feA3C	10	2/3	0.08	8	1	1	0.2	0.1	0.03
feA3H	10	70/9	1.22	1	4	16	7	4.0	1.08
feA3CH	10	99/5	1.60	0	4	17	9.9	5.6	1.52

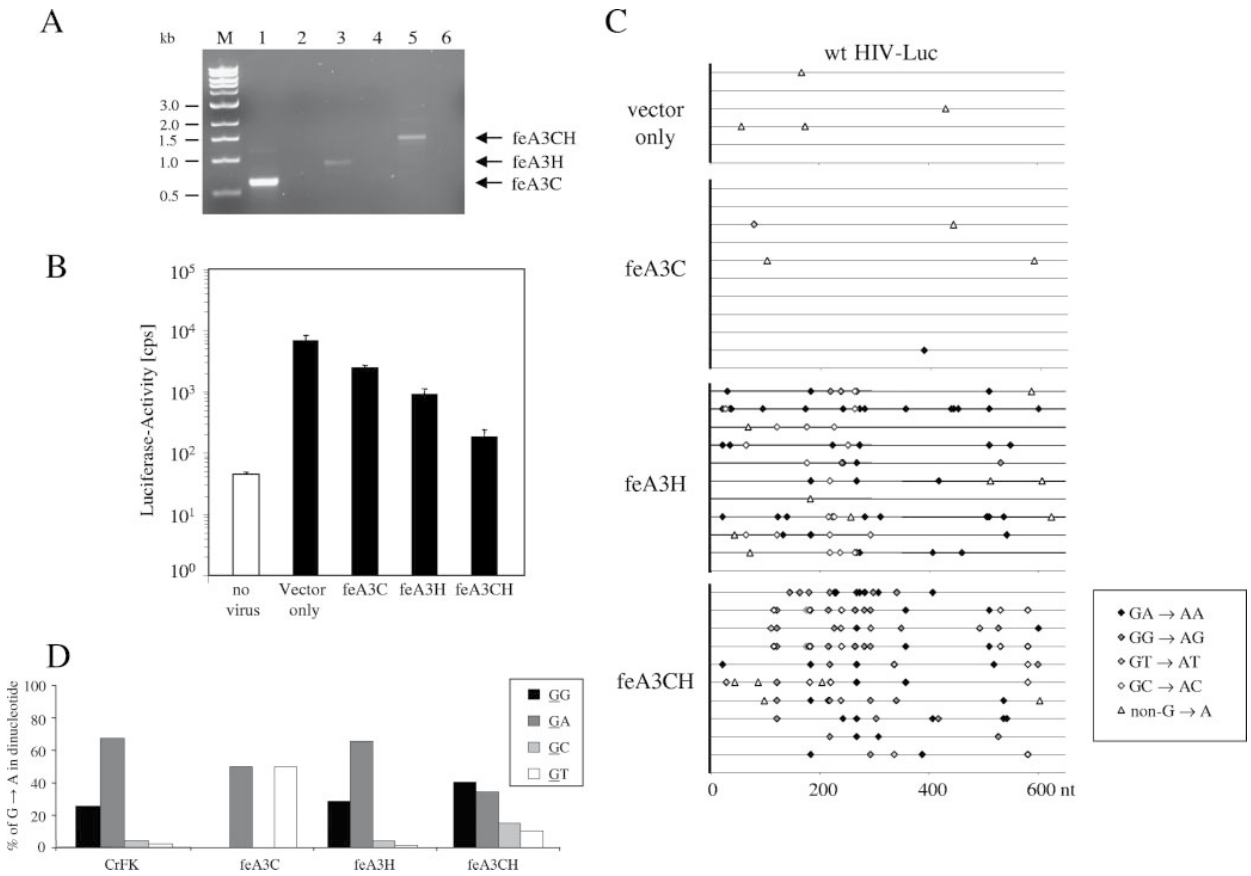

FIG. 6. Defined feline APOBEC3 proteins inhibit HIV-1 infectivity to different degrees. (A) Analysis of feline APOBEC3C (feA3C; lane 1), APOBEC3H (feA3H; lane 3), and APOBEC3CH (feA3CH; lane 5) expression by RT-PCR of total RNA from feline CrFK cells. In lanes 2, 4, and 6, PCRs using the primers specific for feA3C (lane 2), feA3H (lane 4), and feA3CH (lane 6) were performed without template cDNA added. (B) HIV-Luc(VSV-G) was produced in 293T cells in the presence or absence of the indicated feline APOBEC3. Infectivity of the viruses was determined by quantification of luciferase activity in HOS cells infected with equal amounts of viruses 3 dpi. (C) A fragment in LTR-gag was amplified from reverse transcripts of HIV-Luc generated in the presence of the indicated feline APOBEC3 10 h postinfection. A total of 5 to 10 independent nucleotide sequences were determined. The mutations in the clones of each group are shown. Each mutation is indicated and coded with respect to nucleotide mutation. (D) Comparison of the dinucleotide sequence context of G (underlined)→A mutations in the positive-strand DNA of HIV-Luc derived from CrFK cells and from feAPOBEC3-expressing 293T cells.

presence of cotransfected feA3 expression plasmids and equal amounts of particles were used for infection experiments. The results depicted in Fig. 6B show that two of the three feline APOBEC3 proteins are inhibitors of HIV-1-mediated gene transfer: feA3H and feA3CH reduced the infectivity of HIV-1 by 7- and 37-fold, respectively. Only marginally reduced titers were observed in experiments using feA3C. The suppression of reporter gene transfer clearly correlated with a significantly increased G→A mutation rate in viral reverse transcription products (Fig. 6C, Table 1): cotransfection of feA3H or feA3CH resulted in 1.08% and 1.52% G→A substitutions, respectively. Viral genomes derived from transfections omitting an feA3 expression plasmid showed no G→A editing; using the feA3C expression plasmid only 0.03% G→A exchanges were detectable (Fig. 6C, Table 1). The sequence context of the majority of the G→A exchanges in viral genomes derived from 293T cells coexpressing feA3H was similar to that seen with CrFK cell-derived particles (Fig. 6D): feA3H induced 28% GG→AG and 65.7% GA→AA exchanges in the positive strand of the DNA; similarly, viral genomes of CrFK cell-released virions showed 25.5% GG→AG and 68% GA→AA mutations. The editing context of the more antiviral feA3CH showed a different distribution of dinucleotides containing G→A exchanges: 40.4% were GG→AG changes, 34% were GA→AG mutations, and GC or GT to AC or AT changes were found in 15% or 10%, respectively. These findings support the idea that the HIV-1 genome editing of CrFK-derived particles may be attributable to feA3H or to a closely related feline cytidine deaminase.

All three feline APOBEC3C proteins were detectable in purified particles of wt and Δ*vif* HIV1-Luc reporter viruses by immunoblotting (Fig. 7B), suggesting that the Vif protein of HIV-1 cannot induce their exclusion from virions as it does with huAPOBEC3G (data not shown). Particles generated in the presence of feA3CH showed in addition to the 53 kDa protein a second smaller protein of ~23 kDa, which was de-

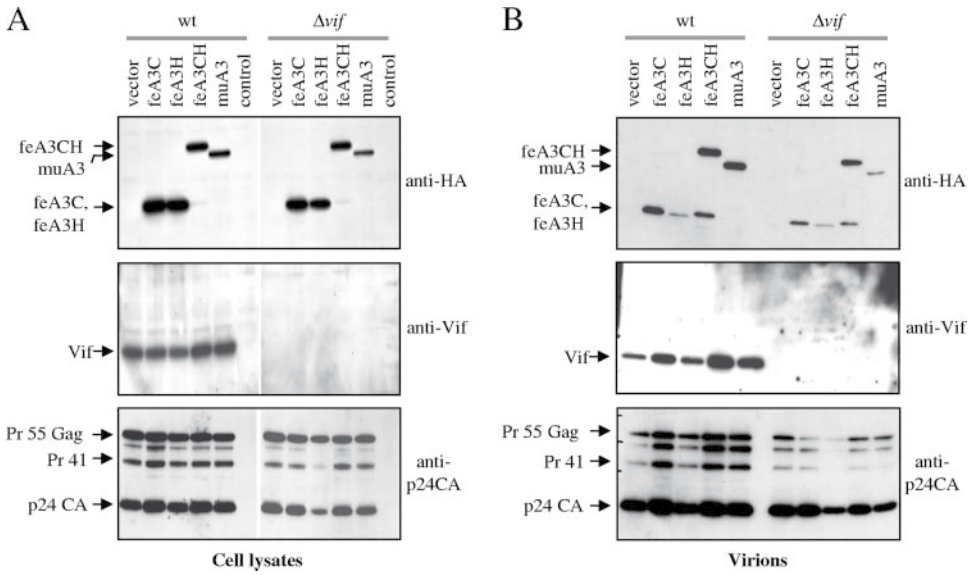

FIG. 7. Feline APOBEC3 proteins are encapsidated into HIV-1 virions. Virions were generated by cotransfection of 293T cells with wt HIV-Luc or Δ*vif* HIV-Luc and HA-tagged feline APOBEC3C (feA3C), APOBEC3H (feA3H), APOBEC3CH (feA3CH), and murine APOBEC3 (muA3) expression plasmids or pcDNA3.1 (vector). (A) APOBEC3 expression in the transfected cells was detected by immunoblotting using anti-HA monoclonal antibody, Vif expression was detected using Vif antiserum, and expression of HIV-1 *gag* proteins (Pr 55 Gag, Pr 41, p24 CA) was detected using p24CA-specific monoclonal antibody. control, lysate of 293T cells not transfected. (B) Encapsidated APOBEC3 in virions normalized by RT activity was detected by probing with anti-HA antibody on a parallel immunoblot. Immunoblots of virions were also probed with Vif antiserum and anti-p24CA monoclonal antibody.

tectable in protein lysates of the producer cells only as a very faint band (compare the results shown for anti-HA in lane fe3ACH of Fig. 7A with those shown for anti-HA in lane feA3CH of Fig. 7B). We do not know whether this 23 kDa protein is generated in the 293T cells and preferentially encapsidated or produced in virus particles by cleavage of the feA3CH with the viral- or a particle-associated protease. In conclusion, it is likely that feline APOBEC3 proteins identical or related to feA3H and eventually to feA3CH significantly contribute to the block of spreading replication of HIV-1 in the CrFK.CD4.CCR5 cells.

DISCUSSION

In this study, we characterized the ability of different feline cell lines to support HIV-1 replication with the long-term aim to use cats (*Felis catus*) or genetically modified cats as an animal model for HIV-1 studies. We found that the expression of functional CD4 and coreceptor molecules in feline cell lines was insufficient to constitute a permissive environment for HIV-1 replication. In fact, additional blocks to HIV-1 are present in feline cells. We identified the feline cytidine deaminases of the APOBEC3 family as one of the factors which exert a strong inhibitory effect on HIV-1 replication in feline cells. To study the transduction of and entry into defined feline cells by HIV-1, single-round, replication-deficient HIV-Luc was used. In these assays with VSV-G-pseudotyped HIV-Luc, two adherent feline cell lines, CrFK and KE-R, were shown to be very permissive towards transduction by HIV vectors: HIV-LTR-driven expression generated a level of luciferase activity as high as in the human reference cell line HOS. In contrast, two feline T-cell lines used showed a restriction, with ~10-fold-lower luciferase counts compared to the human T-cell line A3.01. Infection with HIV-$1_{NL\text{-}BaL}$(VSV-G) followed by immunoblotting revealed a strong correlation of the expression of the luciferase reporter and viral Gag proteins: the feline adherent cells showed high expression of Gag-Pol, whereas, in contrast, the feline T-cell lines expressed low levels of Gag-Pol. Using FIV-based reporter vectors, we could show that the VSV-G-mediated entry pathway for lentiviruses works equally in human and feline T-cell lines, ruling out a trap of the HIV(VSV-G) particles in endosomal vesicles. Because HIV vectors expressing an EGFP gene driven by an internal CMV promoter were, like HIV-Luc, also clearly restricted in the feline T cells, our data suggest that the restriction to HIV-1(VSV-G) virions is not because of VSV-G pseudotyping and LTR-driven transcription but is a cytoplasmic postentry block due to the presence of HIV proteins. This observation is reminiscent of studies of HIV-1 restriction in rodent cells. In transgenic rat lymphocytes, HIV-1-luciferase levels were three- to sevenfold lower than those seen in parallel infections of primary human T lymphocytes (29). Baumann et al. (3) reported that murine T-cell lines had a nonsaturable block at an early, postentry replication stage which was not present in murine fibroblastic cell lines. While murine and feline T cells are more restrictive towards HIV-1 replication than fibroblastic cell lines, studies of simian cells also revealed a postentry restriction in fibroblastic cells, which was identified as an early satu-

rable block at reverse transcription (5, 10, 53). The discovery of the cytoplasmic-body protein TRIM5α was a milestone in characterizing this cellular inhibitory activity in simian cells (72). It is currently unknown whether members of the *Felidae* carry a TRIM5 gene. Interestingly, however, *Mus musculus* seems to be devoid of it. Since the adherent feline cells did not show a TRIM5α-like restriction to HIV-1, the HIV-1 restriction in the feline T-cell lines could be TRIM5α dependent only if TRIM5α or a different inhibitor was expressed in a cell type-specific way. Although feline and rodent T cells have a postentry restriction for HIV-1 in common, we do not know whether the underlying mechanisms for this reduced susceptibility are related to each other. The involvement of a TRIM5α-like protein in the restriction of HIV-1 in feline T-cell lines is possible, but the involvement of a missing factor cannot be ruled out.

HIV-1 can enter cells expressing CD4 and CCR5 molecules of human and simian origin (15). We generated feline CrFK and KE-R cells expressing huCD4 and huCCR5. HIV-Luc viruses pseudotyped with HIV-Env efficiently transduced these cells, indicating that the human proteins are functional as HIV receptors or coreceptors in feline cells. In addition, we used HIV-1 to test the ability of feline CD4 in human cells to support entry of CXCR4 or CCR5. Our results suggest that feline CD4, in similarity to mouse and rabbit CD4 molecules (22, 33), cannot support the entry of HIV-1. Willett et al. (80) reported that feline CXCR4 functions as a coreceptor for Env-mediated cell fusion for the HIV-1 strain LAI. In our study we did not address the role of feline CCR5 or CXCR4, mostly because an antibody against feCCR5 was not available and attempts to detect feCXCR4 at the cell surface were unsuccessful.

Expression of huCD4 or huCCR5 did not render the feline CrFK or KE-R cells susceptible to spreading replication of CCR5-tropic HIV-1. In contrast to results seen with CrFK cells, KE-R cells in repeated experiments showed a profound block in HIV-1 particle release, suggesting differential expression of required and/or inhibitory cellular factors. But in contrast to the results previously obtained regarding the assembly and release block of HIV-1 in murine cells (6, 45), KE-R cells efficiently processed the Gag precursor. In primate cells, a similar cell type- and species-specific block in the release of progeny virions was described as dependent on the presence or absence of the viral *vpu* gene. Vpu facilitates the release of particles. Some cells are "permissive" and others are "nonpermissive" of viral replication in the absence of Vpu (18, 67, 68). In nonpermissive cells, Vpu is considered to counteract a dominant-negative factor whose identity remains to be determined (56, 77). It is possible that levels and/or species-specific variants of the ion channel TASK-1 are responsible for the block in release of virions in primate and feline cells (27). Clearly, more studies of the HIV-1 release block in KE-R cells are required to support or rule out the relevance of Vpu for HIV-1 replication in feline cells.

While the feline CrFK cells efficiently released HIV-1 particles, these virions showed a strongly suppressed level of infectivity. The viral cDNAs of these virions were mutated and preferentially showed G→A mutations, which are typically found when APOBEC3 proteins are encapsidated into retroviral particles, transferred into the newly infected cell, and deaminate single-stranded reverse transcription intermediates (8, 24, 34, 39, 42, 43, 82, 84). HIV-1 Vif can only counteract APOBEC3 cytidine deaminases after species-specific molecular interactions (43). In consequence, HIV-1 is able to replicate in the presence of the human APOBEC3G but is strongly suppressed by the orthologous proteins of African green monkeys or mice (43). Therefore, we analyzed the effect of the activity of three CrFK cell-derived feline APOBEC3s on the infectivity of HIV-1. We found that feA3C, a potent inhibitor of FFV (39), showed only a mild inhibitory effect on HIV-1. In contrast, feA3H and feA3CH were strong inhibitors of HIV-1. Only feA3H and feA3CH induced significant number of G→A mutations in the viral cDNA during the infection of target cells. The feA3H and feA3CH proteins were encapsidated in HIV particles, suggesting that the HIV-Vif protein does not bind and exclude them from released virions or that it targets them for proteasome-mediated destruction (compare the results shown for anti-HA in the wt lanes in Fig. 7B with the results shown in the Δ*vif* lanes). Interestingly, feA3C is also packaged into HIV particles but fails to mutate the HIV genome. A very similar observation was described for the huAPOBEC3C, which is also encapsidated by HIV-1 but does not reduce its infectivity (82). Currently we are characterizing the retrovirus restriction activity of the feline APOBEC3s by use of FIV-, FFV-, and feline leukemia virus-based reporter systems.

The development of a transgenic animal model permissive for HIV-1 infection would aid studies of HIV transmission and pathogenesis and would allow testing of therapeutic strategies that include vaccines. A major obstacle to this end has been the inability of cells from present transgenic rabbit or rodent models to support a robust productive HIV-1 infection (6, 11, 45). Our study extents the list of multiple restriction mechanisms in nonhuman cells by describing the presence of antiretroviral factors in feline cells. In particular, components of the genome-deaminating machinery have been identified and characterized in this study. A detailed analysis of how FIV can overcome the cellular barriers active against HIV will likely generate knowledge showing how to genetically adjust HIV-1 to feline cells.

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CHAPTER 3

Functions, Structure, and Read-through Alternative Splicing of Feline APOBEC3 Genes

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BACKGROUND: Over the past years a variety of host restriction genes have been identified in human and mammals that modulate retrovirus infectivity, replication, assembly, and/or cross-species transmission. Among these host-encoded restriction factors, the APOBEC3 (A3; apolipoprotein B mRNA-editing catalytic polypeptide 3) proteins are potent inhibitors of retroviruses and retrotransposons. While primates encode seven of these genes (A3A to A3H), rodents carry only a single A3 gene.

RESULTS: Here we identified and characterized several A3 genes in the genome of domestic cat (*Felis catus*) by analyzing the genomic A3 locus. The cat genome presents one A3H gene and three very similar A3C genes (a-c), probably generated after two consecutive gene duplications. In addition to these four one-domain A3 proteins, a fifth A3, designated A3CH, is expressed by readthrough alternative splicing. Specific feline A3 proteins selectively inactivated only defined genera of feline retroviruses: Bet-deficient feline foamy virus was mainly inactivated by feA3Ca, feA3Cb, and feA3Cc, while feA3H and feA3CH were only weakly active. The infectivity of Vif-deficient feline immunodeficiency virus and feline leukemia virus was reduced only by feA3H and feA3CH, but not by any of the feA3Cs. Within Felidae, A3C sequences show significant adaptive selection, but unexpectedly, the A3H sequences present more sites that are under purifying selection.

CONCLUSION: Our data support a complex evolutionary history of expansion, divergence, selection and individual extinction of antiviral A3 genes that parallels the early evolution of Placentalia, becoming more intricate in taxa in which the arms race between host and retroviruses is harsher.

The domestic cat (*Felis catus*) is an established animal model for studies of the brain, genetics, pharmacology, and nutrition [1]. In addition, the cat serves as a model for viral infectious diseases. For instance, since feline immunodeficiency virus (FIV) shares many features in common with human immunodeficiency virus (HIV), FIV-infected cats serve as an important model for HIV/AIDS, for example, with respect to therapy, vaccination and pathogenesis [2]. In addition, two other exogenous retroviruses are prevalent in cats, with very different outcomes of infection. Feline leukemia virus (FeLV) is a serious oncogenic pathogen of cats [3] whereas feline foamy virus (FFV) has not been firmly linked to any disease [4] and shows potential as a gene transfer vehicle for cats [5]. FIV is endemic to at least 21 free ranging Felidae species, including lion, cheetah, and puma as well as domestic cat [6], while the prevalence of other feline viruses is less characterized. Although molecular and genetic features of these feline retroviruses have been unraveled over the past years, studies on the contribution of host genes in permissiveness towards virus replication and especially in actively restricting virus multiplication, determining disease, and influencing spread and transmission are only now becoming possible due to new achievements in genomics. Recently, the lightly covered whole genome shotgun (WGS) sequences of the domestic cat (1.9× genome coverage) were assembled and annotated based on the comparison with conserved sequence blocks of the genome sequences of human and dog [7]. The detailed upcoming 7× WGS sequence and analysis of the feline genome will provide an important mammalian comparative genome sequence relative to primates (human and chimpanzee), rodents (mouse and rat), and carnivores (cat and dog) and will likely provide new insights into disease inheritance and the relationship between genetic background of the host and infectious diseases.

The APOBEC3 (A3; for apolipoprotein B mRNA-editing catalyticpolypeptide 3) genes are of particular interest because they form part of the intrinsic immunity against retroviruses (for a review see [8]), are under a high adaptive selection [9], and might have undergone a relatively recent unique evolutionary expansion in primates [10]. In humans, A3F and A3G specifically are capable of terminally editing HIV-1 by deamination of cytidine to uracil during reverse transcription in addition to other, still ill-defined antiviral activities [11]. However, the virion infectivity factor (Vif) of HIV actively counteracts this host-mediated restriction [12-16]. The interaction between Vif and A3 proteins is species-specific and may thus limit cross-species virus transmission [17]. Similar

editing has been implicated in the replication of a number of viruses, including simian immunodeficiency virus (SIV), FFV, FIV and hepatitis B virus [18-21]. While foamy retroviruses also utilize an accessory viral protein (Bet) to counteract A3 inactivation, other viruses like human T-cell leukemia virus have evolved *vif*-independent mechanisms to evade A3-mediated restriction, underpinning the importance of this host restriction [21-23].

Our objective was to identify and characterize A3 genes in the feline genome and compare them to those in the human and dog genomes. Fosmid clones used for the 1.9× WGS cat genome project and the accompanying data were organized into a database that could be used for targeted sequencing of regions underrepresented in the 1.9× genome sequence of the cat. We have used this resource to characterize the feline A3 region and to infer its evolutionary history. Our results reveal that, within Felinae, the A3 locus underwent a unique triplication of the A3C gene, whereas the A3H gene exists as a single copy. In addition, we found a gene read-through generating a double-domain A3CH protein. APOBEC3 proteins of the cat are active inhibitors of various feline retroviruses and show differential target specificity.

RESULTS

Recently we described an antiviral cytidine deaminase of the A3 family in cells of the domestic cat [21]. Feline A3 (feA3) was found to be an active inhibitor of *bet*-deficient FFV [21] and SIV (data not shown and see below), but failed to show antiviral activity against wild-type or Δ*vif* FIV (data not shown and see below). However, the presence of a *vif* gene in the FIV genome, assumed to counteract the anti-viral activity of A3 proteins, strongly argues for additional feline APOBEC3s in the cat. This prompted us to search the genome of *F. catus* more thoroughly for A3 genes. Initial attempts to clone cat A3 cDNAs by a combination of PCR and 5' and 3' rapid amplification of cDNA ends (RACE) detected, in addition to feA3, at least two more A3-related RNAs in the feline cell line CrFK [24].

Genomic organization of the feline APOBEC3 locus

Comparative genomic analysis has shown that the genome of the domestic cat contains gene sequences orthologous to AICDA (activation-induced cytidine deaminase; also known as AID) and APOBEC1, 2, 3 and 4 in that they map to syntenic chromosomal regions of human and dog. The chromosomal localizations of APOBEC1, 2, 3 and 4, were determined on cat chromosomes B4, B2, B4, and F1, respectively. To further identify and characterize A3 genes in the annotated Abyssinian cat genome sequence, we studied fosmids that had been end-sequenced as part of the 1.9× domestic cat genome project from Agencourt Bioscience Corporation. To establish a web-based fosmid cloning system, the 1,806 fosmid 384-well plates were stored in assigned locations. A fosmid database of 1,288,606 fosmid clones, sequence-trace IDs, plate and well IDs, and freezer location IDs was generated and linked to the GARFIELD browser and the National Center for Biotechnology Information (NCBI) trace IDs. In this system, fosmid cloning is achieved by using potential orthologues (that is, human, mouse, dog or yeast) of genes of interest and searching for fosmid trace IDs by gene ID/symbol in the GARFIELD browser or by discontinuous MEGABLAST of orthologous sequences to *F. catus* WGS at the NCBI BLAST site. With the trace ID, the fosmid freezer location ID can be retrieved from the fosmid database. We have tested 704 fosmids and could identify with a 99% accuracy 616 of them (87.5%), as confirmed by fosmid end-sequencing. Using this system, we selected a total of seven fosmids that were predicted to encompass A3 genes and three clones were subsequently analyzed by nucleotide sequencing (Figure 1)

Figure 1a shows a percent identity alignment of the 50 kb A3 region sequenced aligned to itself. Gene modeling studies using the predicted nucleotide and amino acid sequences of cat A3 and A3H cDNAs and the programs Spidey [25] and Genewise [26] demonstrated the presence of three feline A3C genes designated A3Ca (identical to A3C cDNA [21]), A3Cb and A3Cc and a single A3H gene arrayed in a head-to-tail formation spanning 32 kb of the 50 kb region sequenced (Figure 1a,b). The A3C genes each consist of four exons with coding sequences that span 3,693, 6,457 and 6,498 bp for A3Cc, A3Ca, and A3Cb, respectively, whereas A3H contains one 5' untranslated exon followed by four coding exons that span 2,237 bp (Figures 1 and 2). Consensus splice acceptor sites were observed for exons 2 to 4 in the three A3C genes and exons 2 to 5 in the A3H gene. Consensus splice donor sites were observed for exons 1 to 4 in A3H and in all four coding exons of the three A3C genes. Interestingly, splicing at the splice donor sites of exon 4 (bold) in all A3C genes eliminates the overlapping termination codon (underlined) of the feA3 cDNA (CTT **AGG T**GA), allowing the generation of chimeric read-through transcripts. Consensus polyadenylation signals (AATAAA) were observed at positions downstream of exon 4 for all three A3C genes - A3Ca (positions 32,505, 33,376 and 33,444), A3Cb (positions 42,083, 42,954 and 43,022), and A3Cc (positions 22,960 and 23,831) - and A3H (position 50,319).

The initially identified cDNA of feA3 (A3Ca) and the coding sequences of the genes A3Cb and A3Cc show 97.6% and 98.9% identical nucleotides, respectively, and 96.3-96.5% identical amino acids to each other. The predicted proteins of A3Cb and A3Cc differ in six or seven amino acids from feA3 (A3Ca; Figure 3; Figure S2 in Additional data file 3). The feline A3C genes show high overall similarity to human A3C with 43.3-43.8% identical amino acids (Figure S2 in Additional data file 3). In addition to the high degree of sequence identity between the coding sequences of the three cat A3C genes, the pattern of repetitive elements, especially in intron 1 of A3Ca and A3Cb (Figure 1A), and self dotplot analyses (Figure 1c) suggested significant sequence identity in noncoding regions of these highly related genes. Supplementary Table 1 in Additional data file 2 shows the size of each intron and the pairwise percent identities between the introns of the three genes: the introns of A3Ca and A3Cb have a high degree of nucleotide sequence identity (98-99%) across all three introns whereas A3Cc shows a lower degree of sequence identity to either A3Ca or A3Cb (67-96%), depending on the size of the intron. Based on the very high similarity of the A3C genes, two gene duplications in rather recent evolutionary times seem to be highly likely. The first duplication yielded

A3Cc and an A3Ca/b progenitor gene. A3Ca/b subsequently duplicated again, resulting in A3Ca and A3Cb. As expected, the cat A3C genes have a more distant relationship to the human A3C group, the feline A3H clusters with the dog A3H gene, but the dog A3A is only distantly related to human A3A (Figure 4a). Double-domain APOBEC3 genes structurally analogous to human A3F or A3G have not been found in the genomes of either cat or dog..

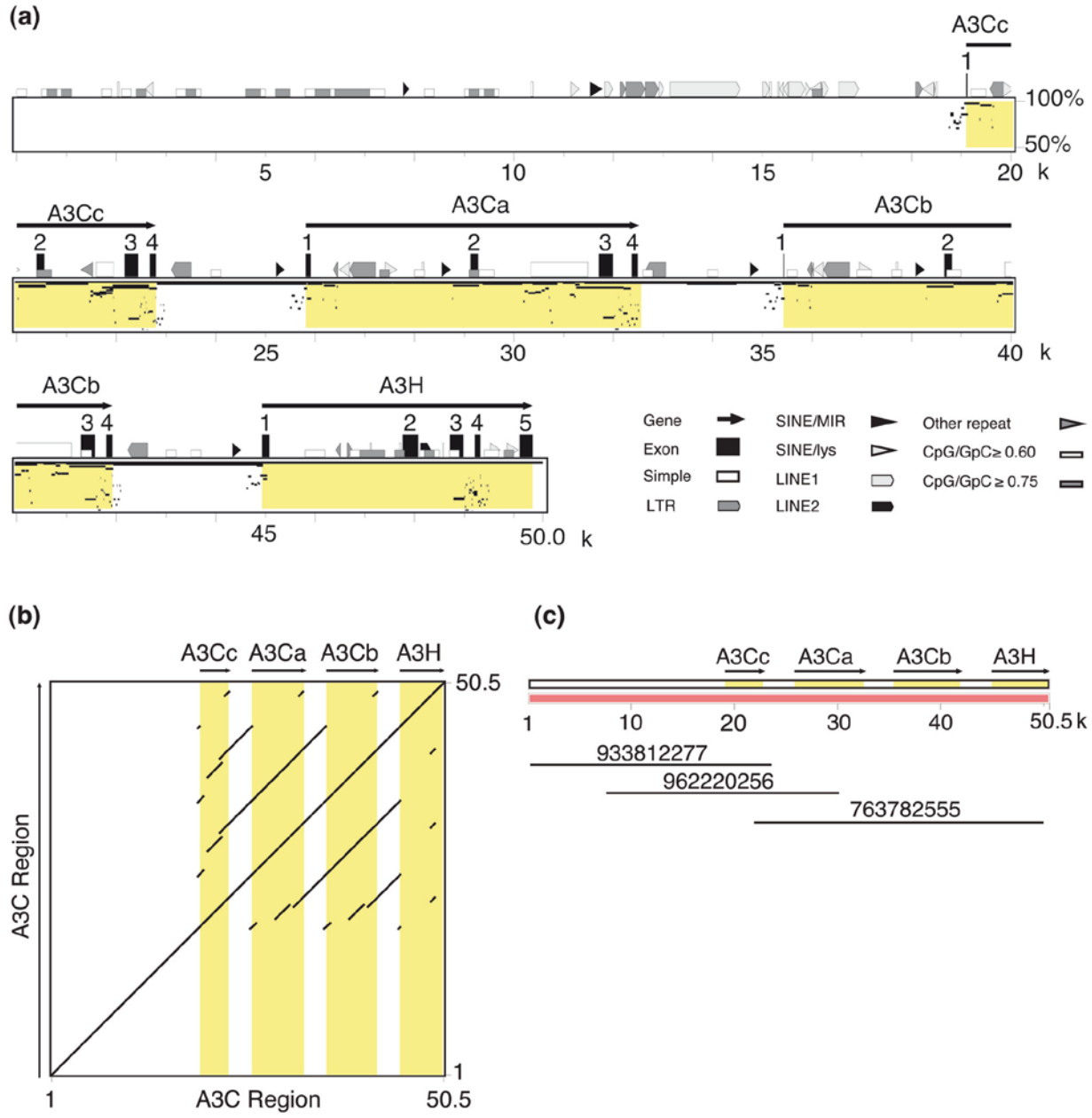

FIG. 1. Gene organization of the feline APOBEC3 region. **(a)** Shown is a Pipmaker analysis of the 50 kb nucleotide sequence of the APOBEC3 region showing the intron/exon organization of the four identified feline A3 genes (A3Cc, A3Ca, A3Cb and A3H) and annotation of repetitive elements (see inset for key: Simple, simple repeat sequence poly(dT-dG).poly(dC-dA); LTR, long terminal repeat retrotransposons; SINE, short interspersed elements; SINE/MIR, MIRs are tRNA-derived SINEs that amplified before the mammalian radiation; SINE/lys, tRNA-lys-derived SINE; LINE1, long interspersed element 1; LINE 2, long interspersed element-2; CpG/GpC ratios are indicated). **(b)** Organization and gene content of the fosmids used for nucleotide sequencing. **(c)** Selfdotplot of the percent identities of the A3C region showing the high degree of sequence identity between A3Cc, A3Ca, and A3Cb.

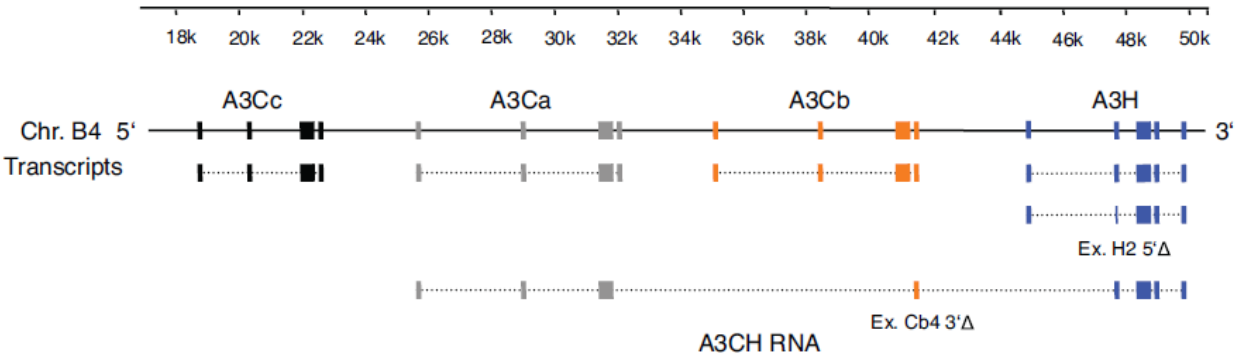

FIG. 2. Representation of the feline APOBEC3 genomic region, portraying the detected A3 transcripts. Transcripts with translated exon (rectangles) and splicedout introns (dotted lines) are indicated. Please note that the transcript for A3H comes in two versions: with complete exon 2 and further spliced exon 2, resulting in 5' truncation (Ex. H2 5'Δ). The mRNA for A3CH includes exon 4 of A3Cb in an additionally spliced version, 3' truncating the sequence one nucleotide before the stop codon (Ex. Cb4 3'Δ).

Expression of feline APOBEC3 genes. Initially, we applied 3' RACE assays using the A3Ca sequence in order to clone additional feline A3 cDNAs. We detected the single-domain A3H and a cDNA composed of the fused open reading frames of A3C and A3H, designated A3CH [24]. A closer inspection of the sequence of A3CH revealed that the transcript is encoded by exons 1-3 of A3Ca, the complete coding sequence of exon 4 of A3Cb and exons 2-5 of A3H (Figure 2). Importantly, the consensus splice donor of exon 4 of A3Cb, located only one nucleotide 5' of the stop codon TGA, is used for in-frame splicing to A3H exons 2-5. The doubledomain A3CH RNA was found in three tested cell lines (CrFK, MYA-1, KE-R) and also in feline peripheral blood mononuclear cells (PBMCs; Figure 5a). In 20 cloned PCR products from independent reverse-transcriptase (RT)-PCR reactions using RNA from CrFK, MYA-1 and PBMCs, the A3CH cDNAs were always exactly as described above (exons 1-3 of A3Ca, the 3' truncated exon 4 of A3Cb and exons 2-5 of A3H). In no case did we observe sequence variation in the A3CH mRNA, for example, by contribution of other A3C exons. We used diagnostic PCR primers to analyze the expression of A3Ca, A3Cb, A3Cc, and A3H in total RNA of feline PBMCs (of two cats of unknown pedigree) and cell lines (CrFK, KE-R, MYA-1). About half of the mRNAs from the activated feline PBMCs corresponded to A3Ca (22 of 40 clones) and approximately 17% were identical with A3Cb (7 of 40 clones) as determined by RT-PCR allowing detection of all three A3Cs. The remaining PCR products of A3C cDNAs represented additional variants, designated A3Cx and A3Cy, each containing six amino acid differences relative to A3Ca (Figure S1 in Additional data file 3), indicating further genetic allelic variation in cats. Sequence-based genotyping by direct PCR of genomic DNA using locus specific primers for exons 2-4 from eight domestic cat breeds resulted in finding zero, thirteen, and four non-synonymous substitutions and zero, one, and two synonymous substitutions in A3Ca, A3Cb and A3Cc genes, respectively (Figure 3b; details in supplementary Table 4 in Additional data file 2). MYA-1 cells expressed A3Ca, A3Cb and A3Cc genes (15, 5 and 1 clone out of 21, respectively), but CrFK and KE-R cells expressed only A3Ca (10 of 10 clones for each). Feline A3H was detected in all analyzed cell lines and PBMCs (Figure 5a). Interestingly, the transcript for A3H seems to be subject to alternative splicing, since we consistently detected an additional variant containing a 5' truncated exon 2, generating a cDNA with a 149 nucleotide shorter 5' untranslated region (Figure 2).

In order to determine whether the different A3 proteins are present in feline CrFK and MYA-1 cells, immunoblot analyses using antisera directed against cat A3C and A3H as well as a serum directed against the A3CH-specific sequence flanked by the C- and H-domains in A3CH (linker) were employed. In extracts from CrFK and MYA-1 cells the anti-linker serum detected a protein band that clearly co-migrated with A3CH expressed from plasmid pcfeA3CH in transfected 293T cells (Figure 5c). The C- and H-domain-specific antisera detected the corresponding A3C and A3H proteins in CrFK cells while only after over-exposition of the immunoblot was the A3CH protein detectable with these sera (data not shown). This detection pattern may reflect low-level expression of A3CH or may indicate that the corresponding epitopes are masked in the two-domain A3CH protein.

To search for transcription factor binding sites that might regulate A3 expression in the domestic cat A3 gene cluster, we first aligned the upstream 1.1 kb, including 100 bp of the predicted exon 1 for each gene A3Ca, A3Cb, A3Cc, and A3H using ClustalW. This analysis showed considerable sequence similarity in the proximal 5' flanking sequences of all four A3 genes, with A3Cc the most divergent (Figure S3 in Additional data file 3). Using MEME to search for conserved sequence elements in a set of DNA sequences using an expectation-maximization algorithm, we detected two highly conserved 50 bp sequence motifs between all four promoter regions, one located flanking the putative transcription start site and the other approximately 200 bp upstream [27,28]. Individual 5' flanking sequences were analyzed using the Match program, which uses a library of nucleotide weight matrices from the TRANSFAC6.0 database for transcription factor binding sites [29]. The first 50 bp motif contains putative transcription fac-

tor binding sites for HNF-4 and Elk-1 as well as a site reportedly present in all phenobarbital-inducible promoters 30 bp upstream of the transcriptional start site. No obvious TATA or CAAT boxes were identified, similar to the human A3 region [30]. The second site (200 bp upstream of the start site) includes Octamer and Evi-1 transcription factor binding sites, which are associated with transcription in hematopoietic cell lineages. Further 5', the sequences and predicted transcription factor binding sites of A3Ca, A3Cb and A3H are relatively well conserved whereas A3Cc is divergent, suggesting that

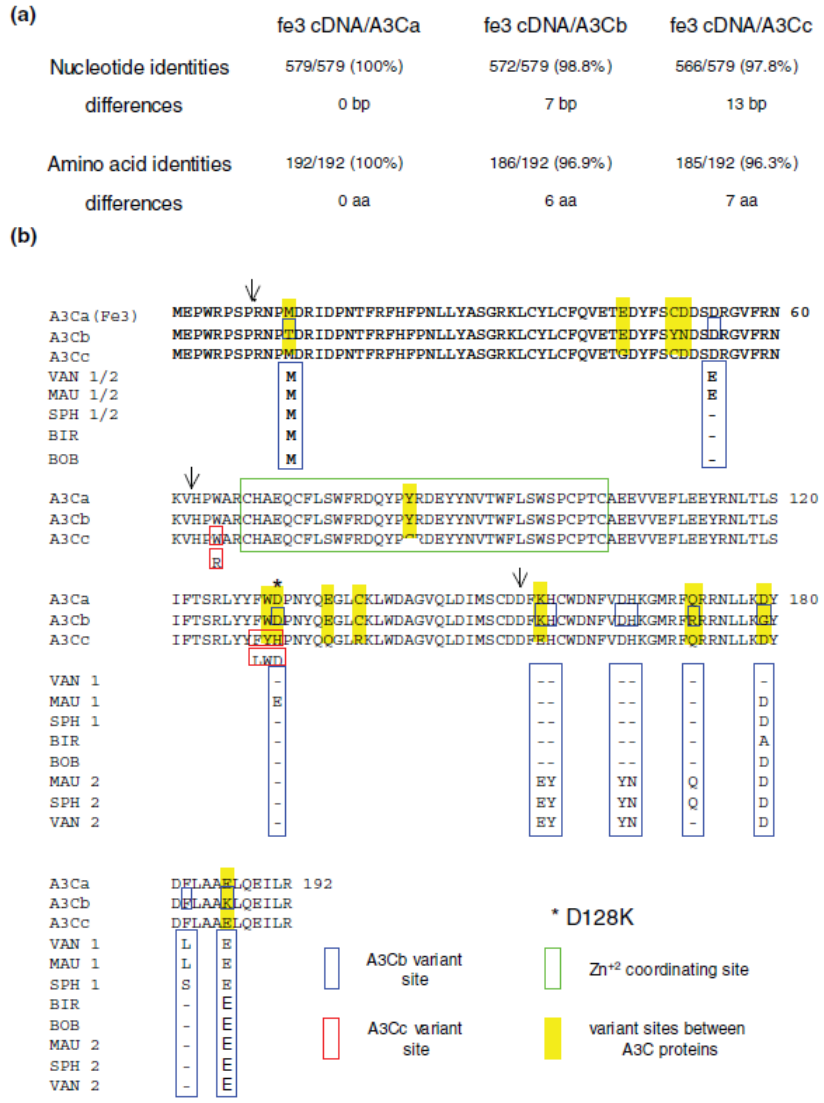

FIG. 3. Comparison of the nucleotide coding and amino acid sequences of the feline A3C genes. **(a)** Pairwise comparison of the domestic cat A3 cDNA to the predicted A3Ca, A3Cb and A3Cc genomic coding sequences and the predicted amino acid sequences. **(b)** Amino acid sequence alignment of A3C cDNA and the predicted proteins for A3C genes. Highlighted in yellow are amino acid residues different between the A3Cs based on the genomic sequence, whereas amino acid sites displaying non-synonymous amino acid substitutions are boxed in blue and red for A3Cb and A3Cc, respectively, as identified by SNP genotyping of eight domestic cat breeds for exons 2-4 of A3Ca, A3Cb and A3Cc (for more details see Table 4 in Additional data file 2). Arrows indicate exonic junctions. Below the alignments, variant amino acids are boxed in red for A3Cc (for example, W65R) and blue for A3Cb with respect to the breed from which they were identified: Turkish van (VAN), Egyptian mau (MAU), Sphynx (SPH), Birman (BIR) and Japanese bobtail (BOB). A dash indicates the amino acid is identical to genomic sequence. Numbers adjacent to breed identifiers refer to alleles 1 and 2 identified by clonal sequence analysis of the PCR products that are in phase for exons 3 and 4, but not for exon 2 (1/2). The residue corresponding to functionally significant amino acid replacement identified in human A3G (D128K) is highlighted by an asterisk (see text).

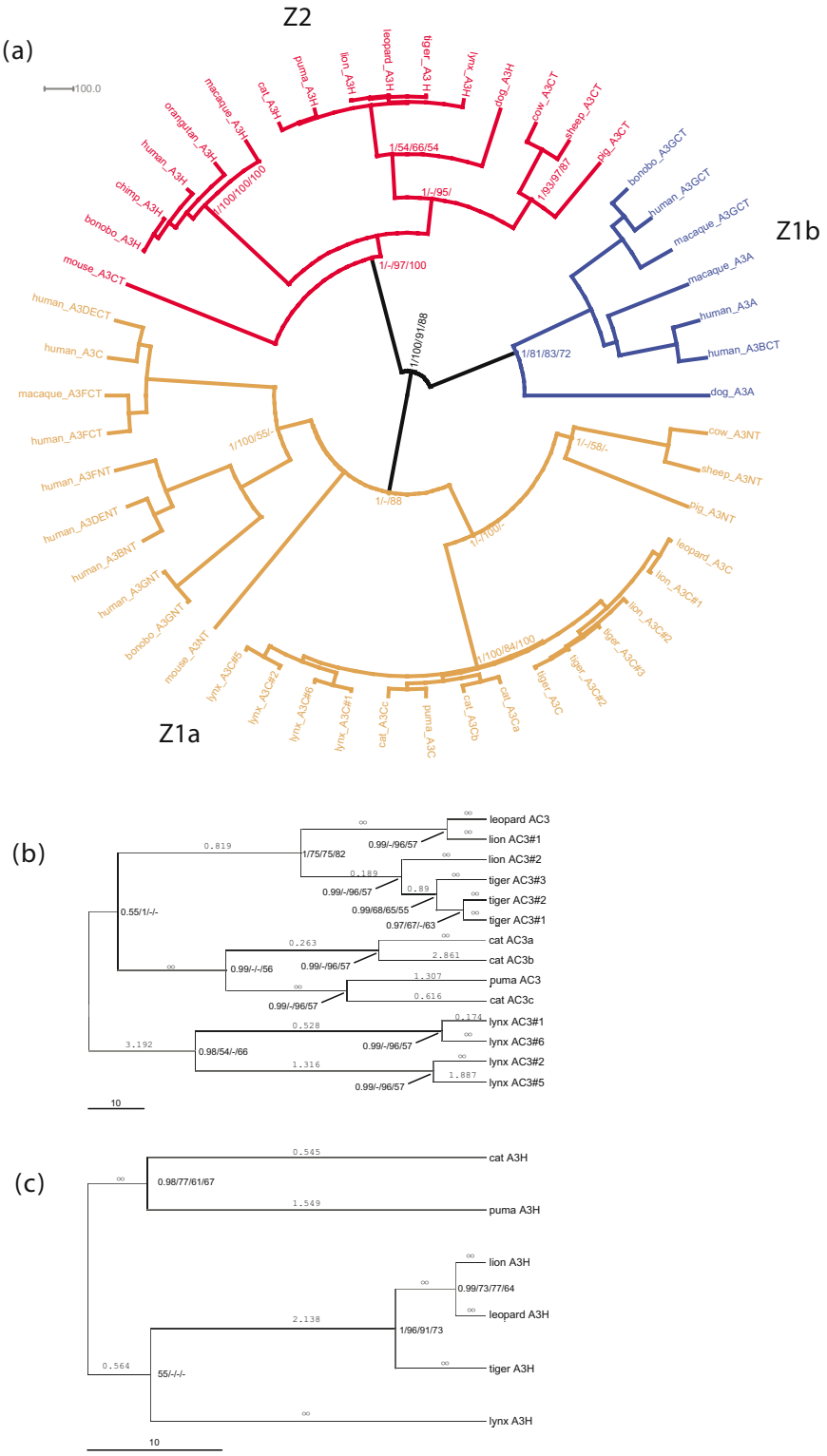

FIG. 4. (see legend on next page)

FIG. 4. (see previous page) Phylogenetic analyses of the feline A3C and A3H genes. **(a)** Maximum clade credibility tree obtained after Bayesian phylogenetic inference with BEAST for the three large clusters of APOBEC3 sequences: A3A, A3C and A3H. Domains in two-domain proteins were split and analyzed separately, their position in the original sequence indicated as CT or NT, for carboxy-terminal or amino-terminal, respectively. Values in the nodes indicate corresponding support, as follows: Bayesian posterior probability/maximum likelihood (percentage after 500 cycles bootstrap)/distance analysis (percentage after 1,000 cycle bootstrap) parsimony analysis (percentage after 1,000 cycles bootstrap). The scale bar is given in substitutions per site. The domains within the A3 proteins can be divided into three groups of related proteins: A3H (Z2), A3A (Z1B) and A3C (Z1A). **(b,c)** Zoom-in on the maximum clade credibility tree obtained after Bayesian phylogenetic inference with BEAST, focusing on the Felinae APOBEC3C sequences (b) and APOBEC3H (c) sequences. Values in the nodes indicate corresponding support, as in the main tree in (a). The scale bar is given in substitutions per site. Figures above the branches indicate Ka/Ks ratios, calculated using Diverge. In some instances, zero synonymous substitutions lead to an apparent Ka/Ks ratio of infinity.

A3Cc has a unique transcription profile as indicated in our RT-PCR expression studies. Another approach to identify transcription factor binding sites, ModelInspector uses a library of experimentally verified promoter modules or models that consist of paired transcription factor binding sites, orientation, order and distance. Using this method, we identified four paired transcription factor binding sites shared between one or more of the feline A3 promoters and that of human A3G [31], including two ETS-SP1 (A3Ca, A3Cb, A3Cc and A3H), IKRS-AP2 (A3H), and NFκB paired with either CEBP (A3Ca and A3Cb), RBPF (A3Ca, A3Cb and A3Cc) or STAT (A3Ca, A3Cb and A3H). Future studies are required to demonstrate the potency of these elements.

Diversity of APOBEC3 in the family Felidae. It was demonstrated that primate A3 genes are under a strongpositive selection predating modern lentiviruses [9,32,33]. Currently, it is not known whether the rapid adaptive selection of A3 genes is unique to primates or represents rather a general feature of Placentalia. To gain further insight into this question, we analyzed A3 sequences of additional Felidae species. We cloned the orthologous cDNAs of A3C and A3H from activated PBMCs of lion (*Panthera leo bleyenberghi*), two tiger subspecies (*Panthera tigris sumatrae* and *Panthera tigris corbetti*), leopard (*Panthera pardus japonensis*), lynx (*Lynx lynx*) and puma (*Puma concolor*). Together with *F. catus*, this collection comprises four of the eight extant lineages within Felidae [34]. We characterized two to six transcripts for A3C and A3H of each species, one animal per species. The phylogenetic relationships and identities to the domestic cat A3 genes are shown in Figure 4b,c, and supplementary Tables 5 and 6 in Additional data file 2. In lynx, lion and tiger, the cDNAs for A3C depicted some degree of intraspecies genetic variability and all variants were included in our analysis. In three of six A3C isolates of Sumatra-tiger and both Indochina-tiger cDNAs, the sequence encoded a lysine at position 185, while in the three other clones of Sumatra-tiger a glutamate was encoded. No further diversity in A3Cc-DNAs of Sumatra-tiger and Indochina-tiger was found. We detected only a single type of A3H transcript in each of the above-mentioned felid species. In Indochina-tiger A3H, we found a polymorphism encoding either an arginine or a lysine at amino acid position 65, whereas in A3H cDNAs of Sumatra-tiger, only K65 was seen. The A3CH transcript was also detected in cDNA preparations of lion, puma, Sumatra-tigerand lynx (leopard was not analyzed) (Figure 5b).

Comparing non-synonymous substitution rates (Ka) and synonymous substitution rates (Ks) within the alignment of the A3C and A3H cDNA sequences, several Ka/Ks ratios were above 1, indicating positive selection among the A3C sequences (Table 2 in Additional data file 2) and the A3H sequences (Table 3 in Additional data file 2) of the different felids. Because extreme Ka/Ks ratios below or above 1 may appear when only few residues are under positive or purifying selection, we used the sliding window approach to determine whether defined regions of the A3 proteins are under any type of selection. The results in Figure S4 in Additional data file 3 show that comparison of feline A3s to the corresponding human A3s do not show clear positive or negative selection as expected due to the evolutionary divergence. In contrast, positive selection of cat, tiger, lion and leopard A3Cs peaks around 200 bp (at the start of the Zn2+-coordinating domain) while comparison with lynx and puma A3Cs reveal different sites under positive selection. In the case of A3H the sliding window comparison was not meaningful because the small number of substitutions led to many infinity values due to Ks = 0. Therefore, the trees of the A3C and A3H genes (Figure 4b,c) were further tested for the presence of selection among amino acid sites using the Phylogenetic Analysis by Maximum Likelihood (PAML) program version 3.15 [35,36]. Evaluating the difference of the maximum likelihood values for the trees calculated with different evolutionary models, a probability estimate for positive selection can be deduced. In the case of A3H the difference is not statistically significant (P = 0.4; Additional data files 1 and 2), but in model 2, which allows for three different ω values (ω = 1 means neutral evolution, ω < 1 purifying selection, ω > 1 positive selection), 71% of A3H are summarized with ω = 0, supporting purifying selection as the simplest evolutionary model. In contrast, positive selection can be found for several residues for A3C sequences (P < 0.0001; 15% of A3C are summarized with ω = 7.2 under model 2). Comparable results were obtained when using the webserver Selecton version 2.2 [37,38] for cat A3Ca and cat A3Cc with the alignment of A3C with all felid species and for cat A3H using the alignment of A3H sequences (data not shown).

The diverse feline APOBEC3s differentially inhibit feline retroviruses. In a recent study we showed that cat A3Ca is a potent inhibitor of *bet*-deficient FFV (FFVΔ*bet*) [21]. We were interested to extend this finding and tested A3Ca, A3Cb, A3Cc, A3H and A3CH as well as dog A3A and A3H with viral reporter systems for FFV, FIV and FeLV. To monitor the activity of the A3s, plasmids expressing hemagglutinin (HA)-tagged versions of

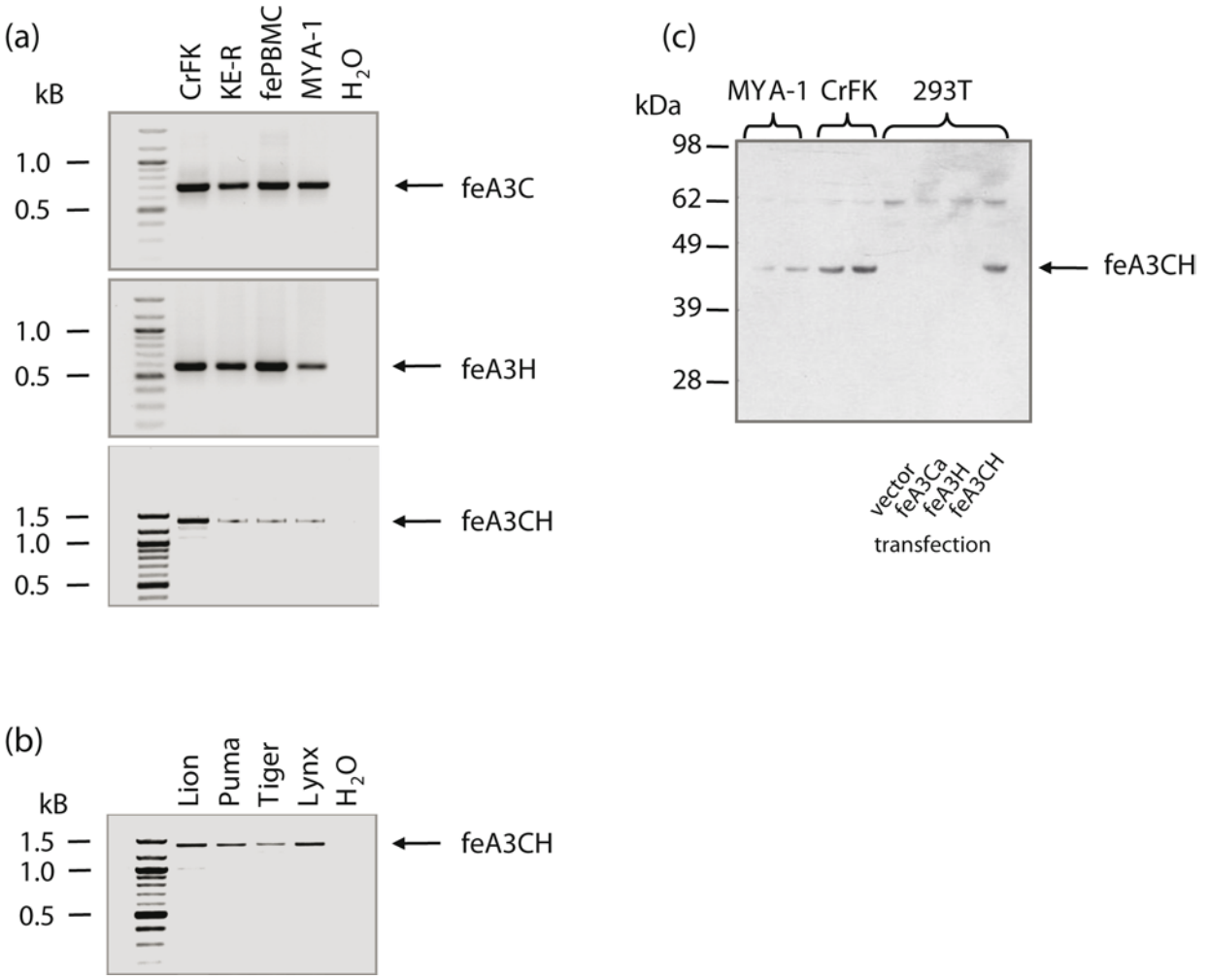

FIG. 5. Expression analysis of feline A3C, A3H and A3CH. **(a,b)** Analysis of expression of feline A3C, A3H and A3CH by RT-PCR of total RNA from feline cell lines (CrFK, MYA-1, KE-R) and feline PBMCs (a) and expression of A3CH in PBMCs of lion, puma, Sumatra-tiger (tiger), and lynx (b). H2O indicates PCRs using primers specific for the A3s without template cDNA added. **(c)** Analysis of expression of cat A3CH by immunoblot using rabbit serum against the sequence flanked by the C- and H-domains in cat A3C (linker) using 293T cells transfected with A3 expression plasmid or empty vector as indicated and CrFK and MyA-1 cells (two independent cultures each).

A3 were used. All A3 proteins could be detected in immunoblots; cat A3Ca, A3Cb, A3Cc and A3CH were comparably expressed, and the expression of cat and human A3H was reduced three- to five-fold (Figure 6a).

The effect of A3 co-expression on wild-type and Bet-deficient FFV was studied after transfection of 293T cells. For this purpose, the infectivity of FFV titers was determined two days after transfection by using FeFAB reporter cells [39]. Cotransfection of A3Ca did not reduce the wild-type FFV titer, whereas a 700-fold reduction in titer was detected with the Bet-deficient FFV (Figure 6b), as described previously [21]. Quite similarly, A3Cb and A3Cc did not inhibit wild-type FFV but reduced the titer of Δ*bet* FFV by 200- and 70-fold, respectively. Feline A3H and A3CH showed a comparable low antiviral activity and reduced Bet-deficient but not wild-type FFV to a much lower degree. Dog A3A and A3H did not inhibit the infectivity of Δ*bet* FFV or wild-type FFV. To assess the antiviral activity of the cat A3s on FIV, vesicular stomatitis virus-G protein (VSV-G) pseudotyped wild-type FIV-luciferase (FIV-Luc), Δ*vif* FIV-Luc and Δ*vif* FIV-Luc cotransfected with Vif expression plasmid (pcFIV.Vif-V5) reporter vectors were generated in 293T cells in the presence of A3 expression plasmids. Equal amounts of particles were used for transduction experiments. The results depicted in Figure 6c show that only two of the five cat A3 proteins are inhibitors of FIVΔ*vif*-mediated gene transfer: feline A3H and A3CH reduced the infectivity by five- and ten-fold, respectively, similar to the human A3H. Feline A3Ca, A3Cb or A3Cc and dog A3A expression plasmids did not reduce infectivity of wild-type or Δ*vif* FIV. In contrast, dog A3H showed antiviral activity against wild-type and Δ*vif* FIV, causing a three-fold reduction. We recently showed that the inactivation of Δ*bet* FFV and HIV-1 by feline A3s was attributable to cytidine deamination of viral reverse transcripts [21]. The suppression of Δ*vif*

FIV by feline A3H and A3CH also correlates with a significant increased G→A mutation rate in the viral genomes (Figure S5a,b in Additional data file 3): cotransfection of feA3H or feA3CH resulted in 1.61% and 1.31% G→A substitutions, respectively. Viral genomes of Δ*vif* FIV derived from transfections omitting an A3 expression plasmid showed no G→A editing; using feA3Ca, feA3Cb or feA3Cc expression plasmids, only 0.1% G→A exchanges were detectable at most. These data highly correlate with the inhibitory activity detected in the infectivity studies. The presence of Vif protein inhibited the genome editing nearly completely (Figure S5 in Additional data file 3). The sequence context of the majority of the G→A exchanges in the viral genomes derived from co-expressing feA3H and feA3CH showed no clear preference for a dinucleotide: feA3H induced 17% GG→AG, 35% GA→AA and 42% GC→AC exchanges in the positive strand of the DNA. The editing context of the A3CH showed 28% GG→AG changes, 39% GA→AG mutations, and 28% GC→AC changes. Both A3s edited in 5-6% GT→AT dinucleotides (Figure S5c in Additional data file 3). Interestingly, in the FIV system, the more antiviral A3CH generated slightly lower numbers of mutations than the less antiviral A3H (Figure S5a,b,d in Additional data file 3). This result could point either to additional and unknown activities of A3 proteins or to differences between the degradation kinetics of uracil-containing DNAs.

To analyze the impact of cat A3 proteins on the infectivity of FeLV, we used a molecular clone of FeLV subgroup A (p61E-FeLV). Reporter particles were generated by co-transfection of the p61E-FeLV packaging construct, a murine leukemia virus (MLV)-based green fluorescent protein (GFP)-reporter genome, a VSV-G pseudotyping plasmid and the different A3 expression plasmids. The FeLV/GFP virions were normalized for RT activity and used for infection of 293T cells. The GFP expression pattern of the inoculated cells demonstrated that cat A3Cs and dog A3H did not reduce the infectivity of FeLV/GFP (Figure 6d). Cat A3H and dog A3A had a marginal effect and A3CH showed a significant effect on FeLV, inhibiting the virus by a factor of 5. We also tested the simian lentivirus SIVagm-LucΔ*vif* and found that all cat A3s, except A3Cc, and dog A3H showed strong antiviral activity. Dog A3A did not reduce the infectivity of SIV (Figure 6e).

In summary, the feline A3Ca, A3Cb, and A3Cc proteins displayed very high activity only against FFVΔ*bet* while A3H and A3CH reduced FFVΔ*bet* infectivity much less. In contrast, only feline A3H and A3CH had a moderate inhibitory effect on Δ*vif* FIV, and A3CH weakly but significantly inhibited FeLV. The Vif protein of FIV counteracted feline A3H and A3CH, but failed to neutralize the antiviral activity of human and canine A3H. The FFV Bet protein mainly counteracted feline A3Ca, as recently shown [21], and A3Cb and A3Cc. We conclude that the various feline A3 proteins differentially target feline retroviruses with a remarkable virus-specific profile.

DISCUSSION

Phylogenetic analysis of the domestic cat APOBEC genes relative to human and dog demonstrated that cat and dog contain genes orthologous to human AICDA (AID), APOBEC1 (A1), A2, A3 and A4. The human A3 gene cluster on chromosome 22 spans 130 kb and contains seven genes that can be classified according to the presence/absence of the Z1a, Z1b and Z2 zinc-coordinating motifs [32,40]. Z1a, the A3C family, consists of human A3C, the carboxy- and amino-terminal domains of human A3DE and A3DF, and the amino-terminal domains of human A3B and A3G (Figure 4a). The Z1b group, the A3A family, contains human A3A and the carboxy-terminal domains of human A3B and A3G. The human A3H represents the Z2 zinc-finger domain. Accordingly, human A3B, A3G, A3DE, and A3F have two domains, while A3A, A3C, and A3H have one domain. Our analysis shows that the genome of the domestic cat contains three A3C genes (A3Ca to A3Cc) in addition to one A3H. The feline A3C genes have a single domain and are related to the human Z1a group but form their own cat specific lineage (Figure 4a). None of the domestic cat genes identified fall into the Z1b group. Cross-species BLAST analyses of the cat 1.9× genome sequence employing dog predicted genes for A3A and A3H using NCBIs cat WGS contig, trace and end-sequence databases failed to identify any cat gene other than A3C and A3H. Presumably, either the cat does not contain Z1b family genes or these genes are not represented in the 1.9× sequence. The fosmid DNA library and database described here provide an additional genomic DNA resource for isolation and characterization of feline genes involved in infectious and inherited disease. Since the fosmids in the library have been mapped to the 1.9× cat genomic sequence by end sequencing, it is not necessary to screen genomic libraries by hybridization or PCR to isolate genes of interest as with previous genomic libraries.

Human A3G and A3F have been shown to be active against HIV-1, which lacks the virion infectivity factor (*vif*) [13-16,41]. The Vif protein of HIV-1 exclusively binds and inactivates human A3 proteins in a species-specific way [17]. The D128K mutation in the human A3G gene altered the Vif interaction [42-44] and H186R correlated with slow AIDS progression in African American populations [45]. In felids three types of exogenous retroviruses are known: within Orthoretroviridae, FIV, a lentivirus related to HIV-1, and the gammaretrovirus FeLV; and, within Spumavirinae, FFV. FIV infects both wild and domestic felid species [6]. Similar to the diversification of SIV in African monkeys and apes, species-specific strains of FIV have been described [46]. But unlike SIV, which is detectable only in African species, FIV is endemic in African, South American and Asian Felidae [6]. For FeLV the prevalence in wild species is not known and limited studies on FFV supported the presence of FFV-related isolates in two species of the leopard cat lineage [47].

The ability of Vif proteins to counteract the antiviral activity of A3 proteins is specific for a virus-host system. Thus, while the HIV-Vif protein counteracts the human A3F and A3G proteins, it is not effective against cat A3H and A3CH, as we recently reported [24]. In contrast, FIV-Vif neutralizes the cat A3H and A3CH induced cytidine deamination. Since we could not detect homologous genes to A3F or A3G in the domestic cat genome, the essential role of controlling retrovirus replication seems to be covered by different A3 proteins in permissive mammals (humans and cats). Interestingly, neither human A3C nor A3H proteins are inhibitors of wild-type or

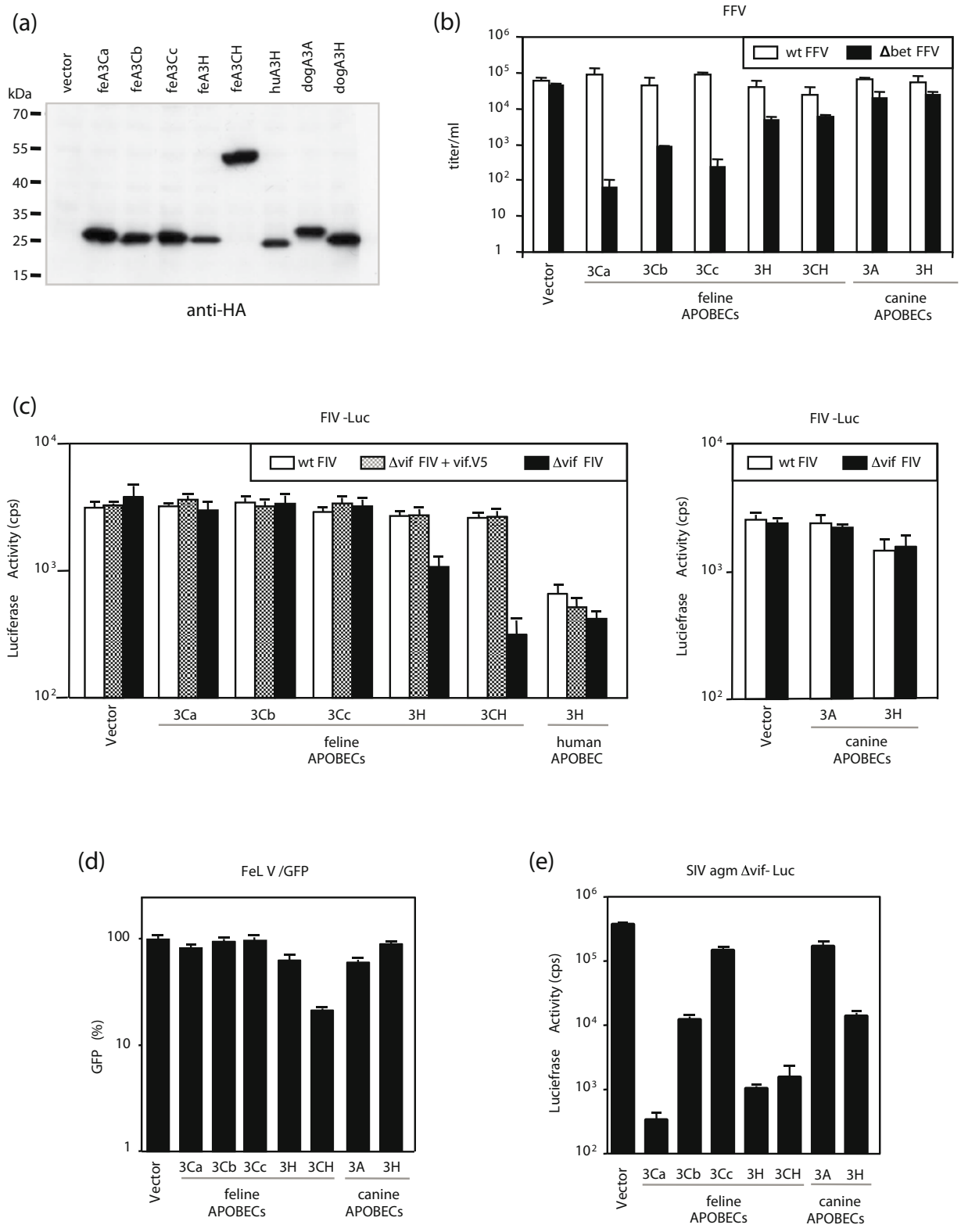

FIG. 6. (see legend on next page)

FIG. 6. (see previous page) Cat A3 proteins selectively inhibit the infectivity of different retroviruses. **(a)** A3 expression in the transfected 293T cells was detected by immunoblottingwith anti-HA monoclonal antibody. **(b-e)** Wild-type (wt) or Δ*bet* FFV wild type (b), wild-type FIV-Luc, Δvif FIV-Luc + Vif expression plasmid (vif.V5) and Δ*vif* FIV luciferase reporter vector particles (c), FeLV/GFP (d), and Δ*vif* SIVagm luciferase viruses (e) were produced in 293T cells in the presence or absence of the indicated APOBEC3s.

Δ*vif* HIV-1 [18,32], supporting a host-specific genetic adaptation of A3 genes.

The *vif* gene of FIV is a relevant modulator of spreading virus infection, since FIV in which the *vif* gene was deleted showed a replication block in feline CrFK cells that express A3, as we showed [21,24,48]. Furthermore, in domestic cats experimentally infected with FIVpco isolated from *P. concolor*, the virus was controlled and the cats did not develop clinical signs associated with FIV infection. The restriction of FIVpco was attributed to feline A3 proteins, because the viral genomes of FIVpco grown in cats accumulated extensive G-to-A mutations [20]. It is likely that insufficient molecular recognition and inactivation of heterologous feline A3 by the Vif protein of FIVpco caused this attenuated virus infection. It is interesting to emphasize here that the *Puma* genus is the closest relative of the *Felis* genus, having diverged approximately 6.7 million years ago [34]. The ability of the cat A3 proteins to limit FIVpco infection while not being able to limit FIV infection may thus reflect the fact that the FIV infecting *F. catus* has evolved the potential to escape A3-mediated restriction of its host since the divergence of both felide lineages. Cat A3H and A3CH also showed some inhibitory activity against FeLV. In contrast to Δ*vif* FIV, these active antiviral proteins showed only weak antiviral activity against Δ*bet* FFV. Based on these findings, we conclude that specific feline A3 proteins selectively recognize and inactivate only defined subgroups of feline retroviruses, while 'non-adapted', heterologous retroviruses (for example, Δ*vif* SIVagm) can be inactivated by all three types of feA3s with the remarkable exception of feA3Cc. These data also reflect the fact that even without expression of Vif or Bet proteins, retroviruses differ, for unknown reasons, in their vulnerability to cognate A3 proteins.

The analysis of the genomic sequences and cDNAs of the cat A3 loci allowed us to identify three key-features not present in the primate A3 system: first, one ancestral A3C gene underwent two successive duplication events in recent times - the first event generated the ancestor of the present A3Cc gene and a second gene, which later on underwent a second duplication giving rise to the ancestors of the present A3Ca and A3Cb genes; second, the A3H gene in domestic cats is under purifying selection; and third, the double-domain A3CH is generated by a read-through transcription and alternative splicing of three genes. In addition, we detected at least 15 single nucleotide polymorphisms (SNPs) yielding non-synonymous substitutions in A3C genes of 8 different cat breeds. In primates, the seven A3 genes (A3A to A3H) are present as single copies on chromosome 22. In the genome of the domestic cat, we found three copies of the A3C gene (A3Ca, A3Cb and A3Cc) in a head-to-tail orientation on chromosome B4. The feline A3C genes encode proteins that are different to each other at six to seven amino acid sites. Phylogenetic analyses indicate that this gene triplication likely occurred by two consecutive duplication steps: one ancestral A3C gene duplicated to the ancestor of A3Cc and a second gene, which later duplicated, giving rise to A3Ca and A3Cb genes. The presence of a homologous A3Cc gene in *P. concolor* closely related to the cat A3Cc gene suggests that at least the first duplication event occurred before the divergence of the *Puma* and *Felis* lineages, approximately 6.7 million years ago. The phylogenetic position of *P. tigris* A3C basal to the three cat A3C genes suggests also that the first duplication event occurred after the divergence of the *Panthera* and the *Felis* lineages, approximately 10.8 million years ago. It is generally believed that the evolution of new protein functions after gene duplication plays an important role in the evolution of the diversity of organisms and typically allows for an increased specialization or function gain of the daughter genes [49,50]. In light of the seven A3 genes in primates, it is tempting to speculate that cats, like primates, were under a specific evolutionary pressure to increase the diversity of the co-expressed A3 proteins that provided additional fitness. Other mammals, such as rodents and eventually dogs, were either not faced by these infectious agents or managed to counteract retroviruses and related retroid elements in a way not involving A3 proteins.

While primate A3 genes are under an adaptive (positive) selection [9,32,33], we detected significant positive selection only for the feline A3C genes. Feline A3H was found to have more residues under purifying selection than feline A3C. It thus appears that restriction against an apparently innocuous virus (FFV), mediated in cat by the A3C genes, is under high selective pressure whereas A3H, which is active against two serious cat pathogens, FIV and FeLV, does not evolve adaptively. While we consider it unlikely that FFV has a strong but currently unidentified pathogenic potential, it is possible that restriction against additional pathogens has shaped this evolutionary pattern. For instance, the cat A3H may protect against highly conserved, endogenous retroelements or may act by targeting highly conserved, invariant viral structures of FIV and FeLV, both features that would result in purifying selection. It could also be that cat A3H took over additional important functions distinct from pathogen defense, inducing purifying selection. Finally, the combination of a conserved A3H domain carrying specifically optimized effecter functions with a highly adaptive module allowing recognition of changing targets may explain that the two-domain feA3CH is much more active against FIV and FeLV than the corresponding single-domain molecules that are either inactive (cat A3C) or have intermediate activity (cat A3H). We postulate that the generation of the fused A3CH transcript is an evolutionary way to gain a greater variety of proteins from a limited number of functional exons.

In order to express the potent anti-retroviral restriction factor A3CH, the cat has modularly combined sequences from A3Ca and A3Cb genes and the constant A3H domain. This was likely achieved by read-through transcription. Read-through transcription, also called transcription-induced chimerism, a mechanism where adjacent genes produce a single, fused RNA transcript, is found in at least 2-5% of human genes

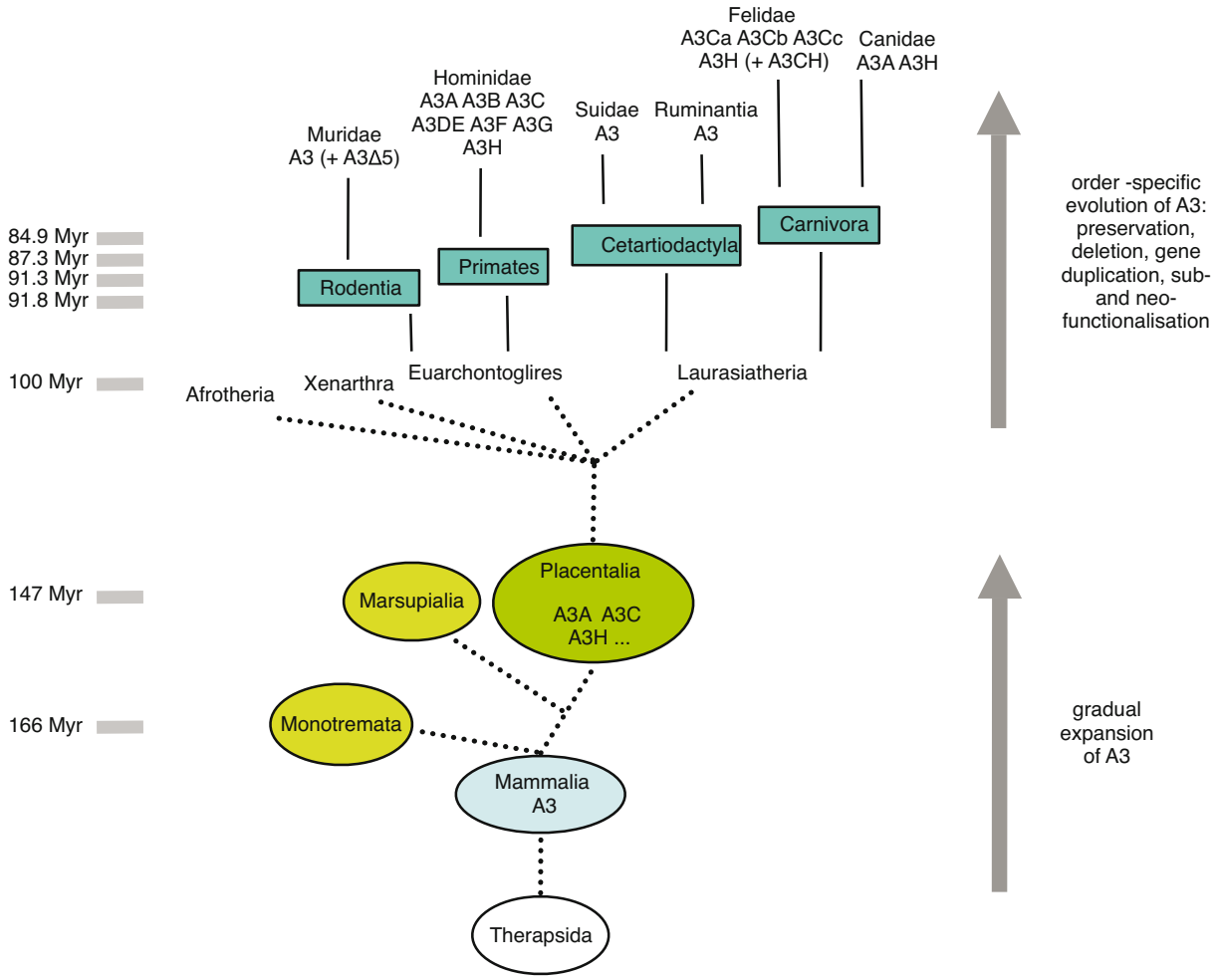

FIG. 7. Model of mammalian A3 gene evolution. The model proposes the presence of several A3 genes in Placentalia before the separation of the super-orders Afrotheria, Xenarthra, Euarchontoglires and Laurasiatheria. According to the phylogenetic relationships among the extant A3 proteins, a host-specific evolution of the A3 genes during the early evolution of the Placentalia orders by means of preservation, deletion and/or gene duplication and concomitant subfunctionalization or neofunctionalization is inferred. Successive duplication events from a single ancestral A3 gene might have generated multiple A3 genes before basal radiation within Placentalia. The divergence times of taxons is in millions of years (Myr) ago. Basal radiation within Monotremata and Marsupialia is not shown. A3, APOBEC3; A3Δ5, A3 lacking exon 5 derived amino acids. Relationship of taxa and timing of mammalian evolution is based on [85], but please note that the timing is controversial [86]. The study of Wible *et al.* [87] supports a later diversification of the placentalia superorders following the Cretaceous-Tertiary (K/T) boundary 65 million years ago.

[51,52]. A general feature of human transcriptional read-through is that intergenic sequences in these RNAs are processed via the standard eukaryotic splicing machinery that removes introns from RNA transcripts. Intergenic splicing is favored in closely located gene pairs [51,52], as true for the triplicated feline A3C genes. Currently, the regulation of read-through transcription is uncharacterized and both *cis*-acting sequences and *trans*-acting suppressors/regulators of the termination machinery could regulate it. Since the cat A3CH protein displayed a significantly stronger antiviral activity against FIV and FeLV compared to the single-domain cat A3C and A3H proteins, the read-through transcription for cat A3 appears to be functionally relevant.

In this study we did not investigate whether the upstream genes (A3Ca and A3Cb) have legitimate transcription termination sites, and whether the downstream gene (A3H) has a legitimate promoter region. But consensus sequences for both regulatory elements are detectable using standard analysis tools (Figure S3 in Additional data file 3). This analysis showed considerable sequence similarity in the proximal 5' flanking sequences of all A3 genes except A3Cc, which has a unique upstream sequence, supporting the experimental data that A3Cc may have a unique transcription profile. In humans, A3 genes are differentially expressed in tissues asso-

ciated with either endogenous or exogenous retroviral replication, including testes, the ovary and un-stimulated and stimulated peripheral blood lymphocytes (for a review, see [53]). Analysis of cDNA clones from domestic cat PBMCs, and MYA-1, CrFK and KE-R cells suggest that the cat A3 genes are also differentially expressed. An alternative possibility, however, is that the fused transcript of A3CH results from *trans*-splicing between separate pre-mRNAs of A3Ca, A3Cb and A3H genes. The amount of trans-splicing in mammals is unknown and only few examples have been described so far [54,55].

In the human A3 locus there is evidence of gene expansion. It was speculated that duplications of single-domain genes formed the two-domain A3B or A3G, and, subsequently, duplication of A3B or A3G formed A3F [30]. Primates and rodents are both part of the placentalia super-order of Euarchontoglires (synonymous with Supraprimates). While primates have seven A3 genes (A3A to A3H), mice and rats carry only a single A3 gene. In mice, in addition, a splice variant lacking exon 5 (A3Δ5) is expressed [17,56]. Based on these data, it was proposed that primates show a relatively recent and possibly unprecedented gene expansion [10] or that the gene expansion happened at the beginning of primate evolution [40]. The lack of data from other mammals was partially filled by Jónsson *et al.* [57], who characterized A3 proteins, designated A3Fs, from certain cetartiodactyla (cow, pig, sheep). Rodent and cetartiodactyla A3 proteins consist of two cytosine deaminase domains, where the amino-terminal domain is similar to human A3C and the carboxy-terminal domain shows the highest identity to human A3H [32]. This CH domain configuration is also found in feline A3CH described here. In our study we detected A3C and A3H genes in Felidae, while in the dog genome only genes corresponding to A3A and A3H are present. Cetartiodactyla and Carnivora are both grouped into the placentalia super-order of Laurasiatheria. Based on the presence of at least three different A3 types in Laurasiatheria, we propose that a certain set of A3 genes (A3A, A3C, A3H, or more) was already established before the separation of the placentalia order. During following evolution, this set of A3 genes was either preserved, fused, deleted or re-expanded depending on the specific requirements of the host-virus interactions (Figure 7). Following this idea, an initial expansion of a single A3 gene happened early in mammalian evolution, eventually before the appearance of the placentalia. Further studies on the absence or presence of A3 genes in the other placentalia super-orders (Xenathra and Afrotheria) and genomics in monotremata and marsupialia are necessary to critically evaluate our model of a two-stage evolution of A3 genes.

CONCLUSION

Recent studies on the evolution of the primate APOBEC3 genes revealed a primate-specific gene amplification. We analyzed the genomic APOBEC3 region of the domestic cat (*F. catus*) and found a chromosomal APOBEC3 locus different to that of primates, rodents and dogs. Besides our detection of three very similar APOBEC3C genes and one APOBEC3H gene, the cat uses the mechanism of transcriptional read-through alternative splicing to generate a fifth antiviral APOBEC3 protein. The evolution of antiviral cytidine deaminases shows a strong placentalia family specific pattern. Our results indicate that three APOBEC3 genes (A, C and H) were present in the evolution of mammals before the placentalia super-orders separated.

MATERIALS AND METHODS

Fosmid database construction and utilization. A fosmid (pCC1) DNA library consisting of 693,504 clones containing an average insert size of approximately 40 kb of domestic cat (breed Abyssinian) genomic DNA arrayed in 1,806 384-well plates frozen at -80°C that was end-sequenced as part of the Feline Genome Project was obtained from Agencourt Bioscience, Inc. (Beverly, MA, USA) The barcoded plates were recorded into a relational database using Filemaker Pro (Filemaker Inc., Santa Clara, CA, USA) according to their rack number and rack position in a -80°C freezer along with individual trace identification number, trace name, and clone identification number (which includes the location of each individual fosmid within the plate).

Fosmid DNA isolation, PCR, and nucleotide sequencing. The appropriate well of a 384-well plate was picked using a culture loop and streaked across LB agar plates containing 12.5 μg/ml chloramphenicol and incubated at 37°C overnight. Fosmid DNA was isolated using a standard alkaline lysis procedure. DNA was diluted 1/1,000 in H2O and assayed by PCR containing 1.0 μM of primer pairs for feline sequence tagged sites, 200 μM deoxynucleoside 5'-triphosphates (dATP, dTTP, dCTP, dGTP), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, and 1 unit TAQ-Gold polymerase in a 20 μl reaction at 95°C for 4 minutes followed by 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s and 72°C for 7 minutes at the end of cycles. PCR products were analyzed on 2% agarose gels containing 0.5× Tris-Borate-EDTA buffer (TBE) and positive PCR products were treated with exonuclease/shrimp alkaline phosphatase and sequenced using BigDye Terminator chemistry with the appropriate forward and reverse primer and analyzed using an ABI 3730XL as described previously [58]. Fosmids were further analyzed by end sequencing and/or transposon insertions. Sequences were assembled using Phred, Phrap, and Consed programs [59-61]. Genomic nucleotide sequences were analyzed using RepeatMasker [62] to identify repetitive elements. Genscan [63], Genewise [26] and Spidey [64] were used to identify coding sequences and Pipmaker [65] to visualize sequence features. Potential regulatory sites, including transcription factor binding sites, splice donor/acceptor sites and polyadenylation sites, were identified using Match™ [66], WebLogo [67,68] and MEME [27,28].

SNP analysis of A3C genes in cat breeds. The following locus specific PCR primers were used for PCR reactions: A3Ca_exon2_F (GCTGTTCTTTGGGGATAGAGAG); A3Cb_exon2_F (GGTTGGGGGTAGGGCGGGCT); A3Cc_exon2_F (CACCCACCAGGGACAACTCG); A3Ca_exon2_R (TGGTTCTCTCCTGGAAACAGA);

A3Cb_exon2_R (AGGCTGTGGCTGGGAGCAGA); A3Cc_exon2_R (TCTGAGAGATCAGAGGGCCG); A3Ca_exon3_4_F (CTCAAAAAAAAAGACAGGGCAGA); A3Cb_exon3_4_F (TCAAAAAAAAAGACAGGGCAGG); A3Cc_exon3_4_F (GATGGATGGATGGATAGATGGAT); A3Ca_exon3_4_R (GCTGGGGAGGGAGGTGCGGA); A3Cb_exon3_4_R (GCTGGGGAGGGAGGTGCGGT); and A3Cc_exon3_4_R (GCTGGGGAGGGAGGTGCGGC). DNA samples for eight domestic cat breeds (Abysinnian, Birman, British Shorthair, Egyptian Mau, Japanese Bobtail, Norwegian Forest, Sphynx, and Turkish van) were amplified by PCR as described above except that we used 25 ng of DNA sample and the annealing temperature was increased to 64-70°C depending on primer pair and DNA sample. PCR products were analyzed as described above and assembled using Sequencher 4.7 (Genecodes, Ann Arbor, MI, USA).

Phylogenetic analysis. Reference sequences for human APOBEC genes and predicted dog APOBEC genes were identified from Ensembl, Refseq and the NCBI annotation of the dog 7× genome sequence, respectively. Domestic cat APOBEC genes were identified using human APOBEC reference gene sequences and were used to screen for traces containing related sequences. Genscan predicted genes were edited and aligned using Seqed and Clustalx (ABCC, NCI-Frederick, Frederick, MD, USA). The sequences used in the phylogenetic trees were aligned using Clustalw [69]. Manual correction of gaps and trimming of the homologous cDNA regions were accomplished with Jalview [70]. For consensus tree construction with bootstrap values, Seqboot (1,000 samples bootstrapping), Dnadist (maximum likelihood distance), Neighbor (UPGMA, jumble 10, different seed values), and Consense from the PHYLIP package [71] were run. The lengths of the branches were calculated with PAML3.15 (59; Model 0, Nssites 2, molecular clock, RateAncestor 1). The ancestral sequences for the different nodes were taken from the PAML result. Alternatively, cDNA sequences were translated and aligned at the amino acid level using MUSCLE [72], manually edited, filtered with GBLOCS [73], and then back-translated conserving the codon structure. Since some APOBEC proteins present two concatenated domains whereas others consist of a single domain, two-domain proteins were split and the individual domains analyzed separately. Parsimony analysis was performed with PROTPARS and with DNAPARS, from the PHYLIP package, after 1,000 cycles bootstrap. Distance analysis was performed using PROTDIST and Dnadist using both neighbor-joining and UPGMA (unweighted pair group method with arithmetic mean), and combining the results. Bayesian phylogenetic inference was performed with BEAST v1.4.6 [74], partitioning the nucleotide sequence following the three codon positions, under the Hasegawa-Kishino-Yano model of evolution, using a strict clock and a Jeffreys prior distribution for the coalescent population size parameter, with no phylogenetic constraints, in two independent chains of 10,000,000 generations, sampling every 1,000 generations. Maximum likelihood analysis were performed with RAxML [75,76] under the Wheland and Goldman model of evolution, executing 500 non-parametric bootstaps.

Ka/Ks analysis of sequence pairs. Ka/Ks values between sequence pairs out of the alignments were calculated using the Diverge program (Wisconsin Package, Accelrys Inc (San Diego, CA, USA). For the sliding window approach (window 300 bp, slide 50 bp) the program was run with the appropriate part of the sequences. Test for positive selection: the cat A3C and A3H trees were further tested for the presence of positive selection among amino acid sites using PAML. The likelihood ratio test was used to compare the evolutionary models M1 (neutral) and M2 (selection), M7 (beta) and M8 (beta&omega variations) [77,78]. The likelihood ratio test statistic was calculated by $2 \times \log\Delta$, where Δ = L0 (null modeldata)/L1 (alternative modeldata), L0 is the likelihood estimate for the simple model, and L1 is the likelihood estimate for the model with more free parameters. The degrees of freedom were determined by the difference in the number of free parameters between the null and alternative models, and the test statistic was approximated to a $\chi 2$ distribution to determine statistical significance. In models M2 and M8, an empirical Bayesian approach is used to calculate the posterior probability that an amino acid site fits in each site class and sites with a high posterior probability of falling into the class of ω of >1 are considered to be under positive selection. Additionally, we identified the residues under positive and purifying selection using the webserver Selecton version 2.2 [37], submitting the alignments of the cat A3C and A3H sequences.

Cells and transfections. Human cell line 293T, and feline cell lines CrFK (ATCC CCL-94, feline kidney cells) and KE-R (feline embryonic fibroblast cells, a gift of Roland Riebe, Friedrich-Loeffler Institut, Riems) were maintained in Dulbecco's high glucose modified Eagle's medium (Biochrom, Berlin, Germany; Dulbecco's modified Eagle's medium complete) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.29 mg/ml L-glutamine, and 100 units/ml penicillin/streptomycin. Feline T-cell lines MYA-1 (ATCC CRL-2417) and FeT-1C (ATCC CRL-11968) were cultured in complete RPMI 1640, 0.29 mg/ml L-glutamine, 10 mM HEPES, and 1.0 mM sodium pyruvate and supplemented with 0.05 mM 2-mercaptoethanol, 100 units/ml human recombinant interleukin-2 and 10% heat-inactivated FBS, and 100 units/ml penicillin/streptomycin. Plasmid transfection into 293T cells was done with Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany). PBMCs of Felidae and a dog were isolated from EDTA- or heparin-treated whole blood by Histopaque-1077 (Sigma, Taufkirchen, Germany) gradient centrifugation and cultured after activation with phytohemagglutinin (PHA; 3 μg/ml) for 3 days in RPMI medium 1640 containing 15% FBS, 5 × 10-5 M 2-mercaptoethanol, 2 mM L-glutamine, and 100 units of human recombinant interleukin-2 per ml at 37°C and 5% CO2. Blood of one dog (*Canis familiaris*) of the breed Australian shepherd was obtained from Karin Kliemann. Blood of *F. catus* was obtained from two cats of unknown breed from the Max Planck Institute for Brain Research, Frankfurt, Germany. Blood from one each of lion (*P. leo bleyenberghi*), tigers (*P. tigris sumatrae* and *P. tigris corbetti*), leopard (*P. pardus japonensis*), lynx (*L. lynx*) and puma (*P. concolor*) were obtained from the Halle Zoo, Germany.

Viruses and infections. FIV single-cycle luciferase vectors (FIV-Luc) were produced by cotransfecting 293T cells with: pFP93 (derived from clone FIV-34TF10, a gift of Eric M Poeschla

[79]), which does not express *vif*; or pCPRΔEnv (derived from clone FIV-PPR, a gift of Garry P Nolan [80]), which does express *vif*; pLinSin; a VSV-G expression plasmid pMD.G [81]; and indicated APOBEC3-HA expression plasmids or empty vector (pcDNA3.1(+) (Invitrogen) or pcDNA3.1(+)zeo (Invitrogen). Vector pLiNSin was derived from pGiNSin, a self-inactivating (Sin) vector variant of pGiNWF [79], which is a minimal bicistronic FIV transfer vector plasmid coding for enhanced-green- fluorescent-protein (EGFP) and neomycin phosphotransferase containing Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) and FIV central DNA flap. The EGFP gene in pGiNSin was replaced by the firefly luciferase gene (luc3) using the restriction sites *Age*I and *Apa*I. The luciferase gene was amplified by overlapping PCR using pSIVagmLuc [17] as template and primers (feLuc3_1.f 5'-TCCACCGGTCGCCACCATGGAAGACGCCAA-3' (*Age*I-restriction site underlined); feLuc3_1.r 5'-CGTTGGCCGCTTTACACGGCGATC-3'; feLuc3_2.f 5'-GATCGCCGTGTAAAGCGGCCAACG-3'; feLuc3_2.r 5'-TTCCGGGCCCTCACATTGCCAAA-3' (*Apa*I-restriction site underlined)), subcloned in pCR4Blunt-TOPO (Invitrogen), and sequence verified. FeLV reporter virions were produced by transfection of 293T cells with FeLV-A clone p61E-FeLV [82], MLV-EGFP transfer vector pMgEGFP-ΔLNGFR [83], VSV-G expression plasmid and the indicated expression plasmid for APOBEC3-HA or pcDNA3.1(+). Reverse-transcription of viruses was determined by Cavidi HS kit Lenti RT or C-type RT (Cavidi Tech, Uppsala, Sweden). For reporter virus infections, HOS cells were seeded at 2.0 × 103 cells/well a day before transduction in 96-well plates and then infected with reporter virus stocks normalized for RT. Firefly luciferase activity was measured three days later with a Steadylite HTS reporter gene assay system (PerkinElmer, Cologne, Germany) according to the manufacturer's directions on a Berthold MicroLumat Plus luminometer. Expression of EGFP was analyzed by flow cytometry. Propagation of wild-type and Bet-deficient FFV (pCF-7 and pCF-Bet-BBtr [21]), contransfection with defined APOBEC3-HA expression plasmids, titration of FFV infectivity, and detection of FFV proteins was done as described previously [21].

Sequencing of viral reverse transcripts. 293T cells (1 × 106) were infected with DNase I (Roche, Mannheim, Germany) treated wild-type or Δ*vif* FIV(VSV-G) (1,000 pg RT) using vector pGiNSin produced in 293T cells together with feline APOBEC3s or pcDNA3.1(+). At 10 h postinfection, cells were washed with phosphate-buffered saline and DNA was isolated using DNeasy DNA isolation kit (Qiagen, Hilden, Germany). A 300 bp fragment of the *egfp* gene was amplified using *Taq* DNA polymerase and the primers eGFP.fw (5'-cgtccaggagcgcaccatcttctt-3') and eGFP.rv (5'-atcgcgcttctcgttggggtcttt-3'). Each of 30 cycles was run at 94° for 30 s, 58° for 1 minute, and 72° for 2 minutes, and PCR products were cloned into TOPO TA-cloning pCR4 vector (Invitrogen) and sequenced. The nucleotide sequences of at least eight independent clones were analyzed.

Plasmids. All APOBEC3s are expressed as carboxy-terminal HA-tagged proteins (APOBEC3-HA). Feline APOBEC3Ca (previously termed feAPOBEC3, feA3, GenBank accession no. AY971954) was described in [21]. Feline APOBEC3Cb and APOBEC3Cc were similarly constructed. Feline APOBEC3H and feline APOBEC3CH cDNAs were identified by using 5' and 3' RACE reactions (5'/3'-RACE kit, Roche Diagnostics) employing total RNA from CrFK cells [24]. APOBEC3 cDNAs of big cats were amplified from cDNA of PBMCs after activation with PHA (3 μg/ml) and *Pwo* polymerase (Roche Diagnostics) was applied. The primers were: for A3C forward primer fApo3F-18 (5'-TAGAAGCTTACCAAGGCTGGCGAGAGGAATGG-3', HindIII site underlined) and reverse primer fAPO3F-19 (5'-AGCTCGAGTCAAGCGTAATCTGGAACATCGTATGGATACCTAAGGATTTCTTGAAGCTCTGC-3' (*Xho*I site underlined)); for A3H, forward primer fAPO-29 (5'-TGCATCGGTACCTGGAGGCAGCCTGGGAGGTG-3' (*Kpn*I site underlined)) and reverse primer fAPO-28 (5'-AGCTCGAGTCAAGCGTAATCTGGAACATCGTATGGATATTCAAGTTTCAAATTTCTGAAG-3' (*Xho*I site underlined)). Thirty cycles were run at 94°C for 30 s, 58°C for 1 minute, and 72°C for 2 minutes. PCR products were cloned into pcDNA3.1(+) using restriction sites *Hin*dIII and *Xho*I (for A3C) or *Kpn*I and *Xho*I (for A3H) and sequenced. Expression plasmids of 3'-HA-tagged dog (*C. familiaris*) APOBEC3A (GenBank accession number. XM_847690.1) and APOBEC3H (GenBank accession number XM_538369.2) were generated by PCR. For dog A3A the template was expressed sequence tag clone nas31ho7 5' (GenBank accession number DN874273), obtained through Graeme Wistow from the National Eye Institute, Bethesda, USA, and *Pwo* polymerase was used. The forward primer was dog-APO-11 (5'-TGCAGGTACCCCGCGGACATGGAGGCTGGCC-3' (*Kpn*I site underlined)), and the reverse primer was Dog-APO-10 (5'-AGTGCGGCCGCTCAAGCGTAATCTGGAACATCGTATGGATATAGGCAGACTGAGGTCCATCC-3' (*Not*I site under-lined)). Dog A3H cDNA was amplified from cDNA of PBMCs after activation with PHA (3 μg/ml), using forward primer Dog-APO-7 (5'-TGCAGGTACCCCACGATGAATCCACTACAAGAAGA-3' (*Kpn*I site underlined)), and reverse primer Dog-APO-8 (5'-AGTGCGGCCGCTCAAGCGTAATCTGGAACATCGTATGGATAAAGTCTCAAATTTCTGAAGTC-3' (*Not*I site underlined)), and *Pwo* polymerase (Roche). Thirty cycles were run at 94°C for 30 s, 58°C for 1 minute, and 72°C for 2 minutes. PCR products were digested by *Kpn*I and *Not*I, introduced into pcDNA3.1(+)-Zeo (Invitrogen) and correct clones identified by sequencing. For generation of the pGex-feAPOBEC3CHlinker the feline A3CH linker region containing the sequence from amino acids 190-244 of the feline A3CH was cloned into a pGEX-4T3 Vector (Amersham Bioscience, Freiburg, Germany) by PCR (forward primer, 5'-CGAGTCGAATTCCCT-TAGTCCCGGCCAACAAAGAAAAAGAGAC-3'; reverse primer, 5'-GCATGAGTCGACTGTGGGTCTGGGCAAGAGGAAGG-3'; the introduced restriction sites for *Eco*RI and *Sal*I are underlined). Purified DNA fragments were fused in frame between the 5' GST domain and the 3' SV40 tag (KPPTPPPEPET) of correspondingly digested pGEX4T3tag derivatives [84]. Clones were identified by restriction enzyme digestion and DNA sequencing. pcFIV.Vif-V5 is an expression plasmid for the codon-optimized *vif* of FIV-34TF10 (GenBank accession number M25381). It was generated by cloning the codon-optimized *vif* gene, 3' fused to the V5-tag, made by Geneart (Regensburg, Germany) into pcDNA3.1(+) using the *Kpn*I and *Not*I restriction sites; a 3'-WPRE element was included in

the *Not*I and *Apa*I sites.

Expression studies. Expression studies of feline APOBEC3C RNAs in CrFK, KE-R, MYA-1, and Fet1C cells and PHA-activated PMBCs of cat and other Felidae were done by RT-PCR using 2 μg of total RNA (RNeasy mini kit, Qiagen) and cDNA made by SuperScript III RT (Invitrogen). For feline APOBEC3Cs the primers were forward fAPO3F-9 (5'-TGGAGGCAGCCTGGGAGGTG-3'), and reverse fAPO3F-15 (5'-GCGAGACGCAAGGAACAGCAG-3'). For feline APOBEC3H the primers were forward fAPO3F-9 and reverse fAPO-26 (5'-CTGCCCGAAGGCACCCTAATTC-3'), or, alternatively, forward feA3H.fw (5'-ATGAATCCACTA-CAGGAAGTCATAT-3') and reverse feA3H.rv (5-TCAT-TCAAGTTTCAAATTTCTGAAG-3'). For feline APOBEC3CH the primers were forward feAPO3CH.fw (5'-ATGGAGCCCT-GGCGCCCCAGCCCAA-3') and reverse fAPO-27 (5'-TCGTACTCGAGGCAGTTTATGAAGCATTGAGATGC-3'). *Pwo* polymerase (Roche) was used for cloning and *Taq* polymerase (Fermentas, St. Leon-Rot, Germany) for diagnostic PCR. PCRs were run for 30 cycles at 94°C for 30 s, annealing for 1 minute at 60°C for feA3C, 58°C for feA3H, and 59°C for feA3CH, and 72°C for 2 minutes. PCR products were cloned in TOPO-vectors (Invitrogen) and sequenced.

Immunoblot analysis. Cells were cotransfected with plasmids for FFV, FIV, FeLV and APOBEC3-HA expression plasmids and lysates were prepared two days later. Cell lysates were prepared by removing the medium from transfected cells, washing them with phosphate-buffered saline, and lysing them in lysis buffer. Protein in the lysates was quantified using Coomassie blue reagent (Bio-Rad, Munich, Germany). Lysates containing 20 μg of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride filters or nitrocellulose membranes. Filters were probed with anti-HA antibody (1:6,000 dilution, MMS-101P; Covance, Münster, Germany), or mouse anti-α-tubulin (1:4,000 dilution, clone B5-1-2, Sigma-Aldrich) or polyclonal rabbit antibody against the linker region of feline A3CH (1:250 dilution) followed by horseradish peroxidaseconjugated rabbit anti-mouse antibody (α-mouse-IgG-HRP, Amersham Biosciences) or Protein A-peroxidase (Sigma) and developed with ECL chemiluminescence reagents (Amersham Biosciences). A polyclonal mono-specific rabbit antiserum against the feline A3CH linker sequence (A3CH amino acid residues 190-244) was generated using a GST-fusion protein made with a pGex-feAPOBEC3CH-linker. The GST-fusion protein was purified as described and used for vaccination of rabbits [21].

Data deposition. The sequences reported here have been deposited in the Gen-Bank database: feline A3 genomic locus, including feA3Ca, feA3Cb, feA3Cc, and feA3H (EU109281); feA3Ca (AY971954); feA3C isolate X (EU057980); feA3C isolate Y (EU057981); feA3H (EU011792); feA3H (Ex2 5'Δ) (EF173020); feA3CH (EF173021); feA1, feA2, feA4, and AICDA (sequences derived from cat genomic project AANG00000000.1); leopard A3C (DQ205650); tiger A3C#1 (DQ093375); tiger A3C#2 (EU016361); tiger A3C#3 (EU016362); lion A3C#1 (EU007543); lion A3C#2 (EU007544); lynx A3C#1 (EU007546); lynx A3C#2 (EU016363); lynx A3C#5 (EU007547); lynx A3C#6 (EU007548); puma A3C (EU007545); leopard A3H (EU007551); tiger A3H (EU007550); lion A3H (EU007549); lynx A3H (EU007553); puma A3H (EU007552); codon optimized *vif* of FIV (EF989123).

ABBREVIATIONS

A3, APOBEC3; AICDA, activation-induced cytidine deaminase (also known as AID; APOBEC3, apolipoprotein B mRNA-editing catalytic polypeptide 3; FBS, fetal bovine serum; FeLV, feline leukemia virus; FFV, feline foamy virus; FIV, feline immunodeficiency virus; HA, hemagglutinin; HIV, human immunodeficiency virus; Luc, luciferase; NCBI, National Center for Biotechnology Information; PAML, Phylogenetic Analysis by Maximum Likelihood; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin; RACE, rapid amplification of cDNA ends; RT-PCR, reverse-transcriptase PCR; SIV, simian immunodeficiency virus; SNP, single nucleotide polymorphism; Vif, viral infectivity factor; VSV-G, vesicular stomatitis virus-G protein; WGS, whole genome shotgun.

AUTHORS' CONTRIBUTIONS

CM, ML and NY designed the study, analyzed data and wrote the manuscript. TB characterized the genomic A3 locus, characterized the SNPs and wrote the manuscript. AHW and IGB performed bioinformatics and wrote the manuscript. JZ, SC and MB performed cell culture and biochemical experiments. KC and SOB contributed material/reagents/analysis tools and analyzed data. JT characterized and provided reagents.

ADDITIONAL DATA FILES

The following additional data are available with the online version of this paper. Additional data file 1 provides supplemental information about the calculation of Ka/Ks. Additional file 2 provides tables showing percent identity of cat A3C introns (supplementary Table 1), Ka/Ks ratios of cat A3s (supplementary Tables 2 and 3), A3C SNPs of cat breeds (supplementary Table 4), percent identities of all described A3C and A3H cDNAs and proteins (supplementary Tables 5 and 6) and results of different evolutionary models (supplementary Table 7). Additional file 3 provides supplementary figures. Supplementary Figure 1 shows amino acid alignments of feline APOBEC3 proteins. Supplementary Figure 2 shows amino acid alignments of feline, canine and human APOBEC3 proteins. Supplementary Figure 3 contains sequences and positions of predicted transcription factor binding sites. Supplementary Figure 4 shows analysis of evolutionary selection by the sliding window approach. Supplementary Figure 5 presents analysis of cytidine deamination of FIV by feline A3s.

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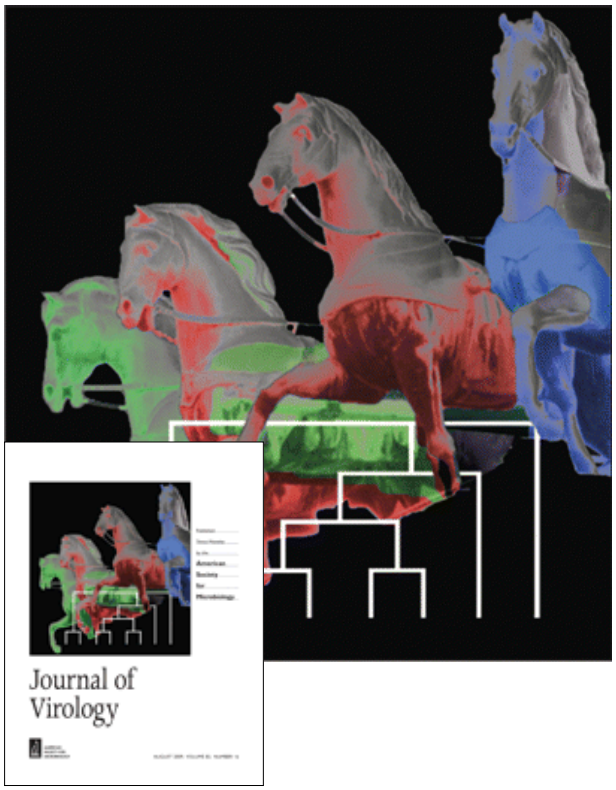
CHAPTER 4

Restriction of Equine Infectious Anemia Virus by Equine APOBEC3 Cytidine Deaminases



Cover photograph (Copyright © 2009, American Society for Microbiology. All Rights Reserved.): The horse *Equus caballus* is the host of the lentivirus *Equine infectious anemia virus*. The antiviral genes of the APOBEC3 family evolved in horses by at least three gene duplications, as indicated by the phylogenetic tree. The genetic variability of these horse genes might modulate the permissivity of individual horses to this pathogenic virus. (*See related article in August 2009, vol. 83, no. 15, p. 7547.*)

Restriction of Equine Infectious Anemia Virus by Equine APOBEC3 Cytidine Deaminases

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The mammalian APOBEC3 (A3) proteins comprise a multigene family of cytidine deaminases that act as potent inhibitors of retroviruses and retrotransposons. The A3 locus on the chromosome 28 of the horse genome contains multiple A3 genes: two copies of A3Z1, five copies of A3Z2, and a single copy of A3Z3, indicating a complex evolution of multiple gene duplications. We have cloned and analyzed for expression the different equine A3 genes and examined as well the subcellular distribution of the corresponding proteins.Additionally, we have tested the functional antiretroviral activity of the equine and of several of the human and nonprimate A3 proteins against the *Equine infectious anemia virus* (EIAV), the *Simian immunodeficiency virus* (SIV), and the *Adeno-associated virus type 2* (AAV-2). Hematopoietic cells of horses express at least five different A3s: A3Z1b, A3Z2a-Z2b, A3Z2c-Z2d, A3Z2e, and A3Z3, whereas circulating macrophages, the natural target of EIAV, express only part of the A3 repertoire. The five A3Z2 tandem copies arose after three consecutive, recent duplication events in the horse lineage, after the split between Equidae and Carnivora. The duplicated genes show different antiviral activities against different viruses: equine A3Z3 and A3Z2c-Z2d are potent inhibitors of EIAV while equine A3Z1b, A3Z2a-Z2b, A3Z2e showed only weak anti-EIAV activity. Equine A3Z1b and A3Z3 restricted AAV and all equine A3s, except A3Z1b, inhibited SIV. We hypothesize that the horse A3 genes are undergoing a process of subfunctionalization in their respective viral specificities, which might provide the evolutionary advantage for keeping five copies of the original gene.

The *Equine infectious anemia virus* (EIAV) (family *Retroviridae*, genus *Lentivirus*) infects equids almost worldwide, causing a persistent infection characterized by recurring viremia, fever, thrombocytopenia, and wasting symptoms (40). EIAV infections are used as a model for natural immunologic control of lentivirus replication and virus persistence and as a test system to improve vaccines against lentiviruses (10, 40, 41, 82). In vivo EIAV replicates predominantly in macrophages (77). Interaction with the equine lentiviral receptor 1 (ELR1) has been demonstrated to be responsible for EIAV internalization (96). There have been no reported cases of EIAV infections in humans, suggesting that it is an intrinsically safe virus and of interest for use in a clinical setting. Therefore, EIAV-based lentiviral vectors for human gene therapy were recently developed (1, 67, 68).

In the last few years, two cellular proteins that inhibit many different retroviruses have been characterized: tripartite motif protein 5 alpha (TRIM5a) and apolipoprotein B mRNA-editing enzyme-catalytic polypeptide 3 (APOBEC3 [A3]) (for a review, see reference 92). With respect to TRIM5a proteins, both human and nonhuman primate TRIM5a orthologues can restrict infection by EIAV (26, 72). The activity of equine TRIM5a on EIAV infection, however, has not been described so far. With respect to A3 proteins, human APOBEC3G (A3G) is the best-characterized member of the A3 gene family. The ability of A3G to restrict EIAV was identified by Mangeat et al. during research focusing on the activity of the *Human immunodeficiency virus type 1* (HIV-1) viral infectivity factor (Vif) protein (48). The mechanism whereby A3G inhibits EIAV was not investigated. HIV-1 mutants lacking Vif package A3G into viral particles. Incorporated A3G specifically deaminates cytosine residues to uracil in growing single-stranded DNA during reverse transcription, leading to HIV genome degradation or hypermutation (5, 25, 39, 48, 49, 98). More recent studies indicate that deaminase-independent mechanisms might also be involved in antiviral activity of A3 (4, 27, 28, 30, 54, 61). The amount of encapsidated A3G in wild-type (wt) HIV-1 virions is dramatically reduced by a Vif-dependent degradation via the ubiquitination-proteasome pathway (50, 79, 94, 95). EIAV is the only extant lentivirus lacking a *vif* gene, a characteristic shared with *Rabbit endogenous lentivirus type K* (34). In contrast to the well-characterized A3-Vif interaction, still little is known about A3-neutralizing strategies used by retroviruses that do not encode a Vif protein. It has been reported that foamy retroviruses use the accessory protein Bet, and *Human T-cell leukemia virus type I* has evolved a unique nucleocapsid protein to counteract the packaging of cognate A3 proteins (12, 44, 58, 71). The debate over the mechanism of resistance to murine A3 (muA3) of the rodent gammaretrovirus *Moloney murine leukemia virus* (Mo-MLV) has not come up with a generally convincing model, despite many studies (5, 9, 13, 32, 35, 49). However, recent data clearly show that muA3 is an important in vivo restriction factor of Mo-MLV and the Friend virus complex (45, 73, 89).

The evolution of the A3 genes appears as a complex, taxon-specific history of expansion, divergence, selection, and individual extinction of antiviral A3 that parallels in some points the early evolution of placental mammals (38, 58). Humans carry seven A3 genes, rodents carry one, pigs carry two, horses carry six, and cats carry four A3 genes (6, 31, 38, 58, 63). For the sake of clarity, the nomenclature for nonprimate A3s has been recently changed, based on the characteristics of the zinc (Z)-coordinating domain, to Z1, Z2, and Z3 (37). In light of

the absence of a *vif* gene in EIAV and of a recent study of Bogerd et al. (6) on two equine A3s (eqA3s) (eqA3Z2a-Z2b and eqA3Z2c-Z2d, previously designated A3F1 and A3F2, respectively) that suggested that EIAV is resistant to eqA3s, we were interested in eqA3 genes and in the differential ability of the eqA3 proteins to restrict EIAV and other viruses.

Here, we identified and characterized five eqA3 proteins in regard to their activity against EIAV, *Simian immunodeficiency virus* (SIV), and *Adeno-associated virus type 2* (AAV-2). In contrast with a previous report (6), we identified eqA3s that strongly inhibited EIAV. The five eqA3s showed differential antiviral specificities against EIAV, SIV, and AAV, which we interpret as evidence for an ongoing subfunctionalization process. These results underpin the possibility that specific A3 paralogues protect against distinct viruses. The process of A3 gene duplication and subfunctionalization might result in an evolutionary advantage, enlarging the antiretroviral armory, and provide an explanation for the expansion of the A3 family in many mammalian species.

MATERIALS AND METHODS

Cells and transfections. The human cell lines 293T, 293, HeLa, and HOS (ATCC CRL-1543); the feline cell line CrFK (ATCC CCL-94); and equine macrophage-like cells EML-3C (20) were maintained in Dulbecco's high-glucose modified Eagle's medium (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 /g/ml). Equine (*Equus caballus*) peripheral blood mononuclear cells (PBMCs) were isolated from heparin-treated whole blood by Histopaque-1077 (Sigma-Aldrich, Taufkirchen, Germany) gradient centrifugation and cultured after activation with phytohemagglutinin (3 μg/ml) for three days in RPMI 1640 medium (Biochrom, Berlin, Germany) containing 10% fetal bovine serum, 5 10_5 M 2-mercaptoethanol, 2 mM L-glutamine, and 100 units of human recombinant interleukin-2 per ml at 37°C and 5% CO_2. Monocyte-derived macrophages were purified from peripheral equine blood following a standard procedure (52). Briefly, monocyte-derived macrophages were purified from peripheral equine blood by gradient centrifugation through Histopaque. Cells were seeded in six-well plates in high-glucose Dulbecco's modified Eagle's medium with 15% horse serum (Biochrom, Berlin, Germany), 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). Nonadherent cells were removed after an overnight incubation, and any remaining adherent cells were incubated for 10 days. Plasmid transfections into 293T cells were performed with Lipofectamine LTX (Invitrogen, Karlsruhe, Germany) according the manufacturer's instructions.

Viruses and infections. EIAV single-cycle luciferase vectors (EIAV-luc) were produced by cotransfecting 293T cells with the following: pONY3.1 (55), pONY8.1luc, the vesicular stomatitis virus G protein (VSV-G) expression plasmid pMD.G (18), and an A3 expression plasmid or empty vector pcDNA3.1(+) (Invitrogen, Karlsruhe, Germany). Vector pONY8.1luc was derived from pONY8.1G (69), a self-inactivating vector variant of pONY8.0 (53) coding for enhanced green fluorescent protein and a deleted *env* region. The *egfp* gene in pONY8.1G was replaced by the firefly luciferase gene (luc3) using restriction sites SacII and NotI. The luciferase gene was amplified by PCR using $pSIV_{agm}Luc$ (where agm indicates the African green monkey SIV host) (49) as a template and the primers Eluc.fw (5'-CCTCCGCGGCCCCAAGCTATGGAAGACGCCAAA-3'; SacII restriction site is underlined; where present, fw indicates forward and rv indicates reverse) and Eluc.rv (5'-AGGCGGCCGCTTTACACGGCGATCTTT-3'; NotI restriction site is underlined), subcloned in pJET1.2/blunt (Fermentas, St. Leon-Rot, Germany), sequence verified, and recloned into pONY8.1 using SacII and NotI restriction sites. The procedure used to generate pONY3.1 *DU* results in an in-frame deletion of 270 bp in the predicted dUTPase (DU) coding region (modified after Threadgill et al. [90]). By using pONY3.1 template DNA and the primer pair EIAV1.fw (5'-GC GCGCGGCGCCTGGCACCACCACAAGGGCCT-3') and EIAV-dDU.rv (5'-T GAGTGCCCTGCATCTTCATCTCT-3') and the pair EIAV2.rv (5'-GCGCGCG AATTCCCATTCCAGTCGTTCCTGG-3') and EIAV-dDU.fw (5'-GCTGGGCA CTCAAATTCCAGACAG-3'), the 5' and 3' fragments were amplified separately. The fragments were fused through overlapping extension PCR and cloned into pONY3.1 using restriction sites KasI and EcoRI. pONY3.1 *S2* was made as described in Mitrophanous et al. (55): the deletion from nucleotide 5345 to 5397 was made by PCR using overlapping primers EIAV-dS2.fw (5'-GTTATAAGGTTTGA TATCAGCAT-3') and EIAV-dS2.rv (5'-CATAGAATGCGATGCTGATATC-3'). SIV reporter virions were produced via transfection of 293T cells with $pSIV_{agm}LucΔvif$, a VSV-G expression plasmid, and an expression plasmid for A3 or pcDNA3.1(). Reverse transcriptase activity of viruses was quantified by using a Cavidi HS Lenti RT kit (Cavidi Tech, Uppsala, Sweden). For reporter virus infections, CrFK, EML-3C, or HOS cells were seeded at 2.0 10_3 cells/well 1 day before transduction in 96-well plates and then infected with reporter virus stocks normalized for reverse transcriptase. Firefly luciferase activity was measured 3 days post-transduction with a Steadylite HTS reporter gene assay system (PerkinElmer, Cologne, Germany), according to the manufacturer's directions, on a Berthold MicroLumat Plus luminometer. To generate recombinant AAV-2, 293 cells were transfected with the *lacZ* reporter plasmid pAAV-lacZ (51, 93), the *rep-cap* encoding plasmid pAAV-RC (51, 93), the adenovirus helper plasmid pHelper (51, 93), and an expression plasmid for A3 or pcDNA3.1(+). Cells were harvested for analysis after 72 h. Lysate containing virions was generated by freeze/thaw cycles, and supernatants were used to transduce fresh 293 cells. Transduction was quantified 3 days later by measuring β-galactosidase activity using a GalactoStar kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions.

A3 expression and plasmids. All A3s are expressed as carboxy-terminal (CT) hemagglutinin (HA)-tagged proteins, except human A3DE (also known as human A3Z2c-Z2d), a gift from Y.-H. Zheng, that carries a CT V5 tag (11). The human A3G and muA3 expression constructs (human A3Z2g-Z1c and muA3Z2-Z3) were provided by N. R. Landau (49). Human A3B, -C, -F, and -H (A3Z2A-Z1b, A3Z2b, A3Z2e-Z2f, and A3Z3); feline A3; and canine A3 expression plasmids were previously described (33, 44, 57, 58). Porcine A3 (A3Z2-Z3) was a gift of Eva Dörrschuck and Ralf To¨njes (GenBank accession no. EU871587; E. Dörrschuck et al., unpublished data). eqA3 cDNAs were identified by using 5' and 3' rapid amplification of cDNA ends reactions (5'/3'-RACE kit; Roche Diagnostics, Mannheim, Germany) employing total RNA from equine PBMCs. For expression plasmids of eqAPOBEC3Z1b (eqA3Z1b), the primers were E-APO-37 (5'-TTCGTGAATTC GGGACTGAGACATGGAGGCC-3'; forward) and E-APO-38 (5'-AGAGCTCG AGTCAAGCGTAATCTGGAACATCGTATGGATACCCAGAGGAGCCTCT GAGTG-3'; reverse); for eqA3Z2e, the primers were E-APO-27 (5'-ACGTGGTA CCTACGTCCAGGCTCCCAGGAAC-3'; forward) and E-APO-28 (5'-TTCTCT CGAGTCAAGCGTAATCTGGAACATCGTATGGATACTTGAGAATGTCC TCAAGCTTTG-3'; reverse); for eqA3Z2a-Z2b and eqA3Z2c-Z2d, the primers were primer E-APO3Ca/c-KpnI.fw (5'-ACGTGGTACCACCCAATGGAGAAGT TGGAT-3'; where present, fw indicates forward and rv indicates reverse) and E-APO3Cb-XhoI.rv (5'-GCGCCTCGAGTCAAGCGTAATCTGGAACATCGT ATGGATACTTGAGAAGGTCCT-3') or E-APO3Cd-XhoI.rv (5'-GCGCCTCG AGTCAAGCGTAATCTGGAACATCGTATGGATACTTGAGAATCT CCT-3'); for eqA3Z3, the primers were E-APO-35 (5'-TTCGTGAATTCGAGGT CACTCTCAGATAAGCGGC-3'; forward) and E-APO-36 (5'-AGAGCTCGAG TCAAGCGTAATCTGGAACATCGTATGGATACCCGAGGTCAACCGACC CAAG-3'; reverse); *Pwo* polymerase (Roche Applied Science, Mannheim, Germany) was used. Each of 30 cycles was run at 94°C for 30 s, 58°C for 1 min, and 72°C for 2 min. PCR products were cloned into pcDNA3.1(+) using restriction sites KpnI or EcoRI and XhoI, and sequences were verified. EA3F2 (6) was made by exchanging amino acids (V40G, P48V, S339I, and K354N) of the eqA3Z2c-Z2d template through overlapping extension PCR, and the resulting cloned PCR product was sequence verified. eqA3Z3 point mutants were generated by exchanging zinc-coordinating amino acids (H84R, C115S, and C118S) or the catalytic glutamic acid residue (E86Q) through overlapping extension PCR. The 5' and 3' fragments were amplified separately by using primer E-APO-35 containing an EcoRI site or the 3' primer E-APO-36 containing an XhoI site. The 5' and 3' fragments were then mixed and amplified with the two external primers. The resulting full-length mutant was cleaved with restriction enzymes and cloned into pcDNA3.1(+) and sequence verified. The human A3F and A3G point mutants have been described previously (7, 23). A3F mutants h3FAS1 (E67A), h3FAS2 (E251A), and h3FAS1/AS2 (E67A/E251A) were a gift of N. R. Landau; the mutant A3G-E259Q was a gift of Barbara Schnierle. Expression studies of eqA3 RNA of PBMCs, macrophages, and spleen cells were done by reverse transcription-PCR (RT-PCR) using total RNA, *Taq* polymerase (Fermentas, St. Leon-Rot, Germany), and the primers E-APO-3 (5'-A GACACCTTCACTGAGAACTTC-3'; forward) and E-APO-2 (5'-ATGGACCAG TAGCGACTCTCA-3'; reverse) for eqA3Z1a and eqA3Z1b; the primers rtE-A3Ce.fw (5'-TTGCATCCCAAGACCTCC-3'; where present, fw indicates forward and rv indicates reverse) and rtE-A3Ce.rv (5'-GGTACTGCTTCATGAAGTCA-3') for eqA3Z2e; the primers rtE-A3Ca.fw (5'-GTGATGTTGCGCAAGGA-3') and rtE-A3Cb.rv (5'-CCCTTGGCAAATTTCAG-3') for eqA3Z2a-Z2b; the primers rtE-A3Cc.fw (5'-TGACACTGGCGGCCA-3') and rtE-A3Cd.rv (5'-TCCGGC TTATTCCTTTGC-3' for eqA3Z2c-Z2d; and the primers E-APO-23 (5'-CAGAC CCACAATGAATCCAC-3'; forward) and E-APO-26 (5'-CTGCTGATTCTGCC TGAACCAG-3'; reverse) for eqA3Z3. The housekeeping gene for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control for RT-PCR using forward primer human GAPDH A (5'-GTTGCCATCAATGA

CCCCTTCATTG-3') and reverse primer human GAPDH B (5'-GCTTCACCAC CTTCTTGATGTCATC-3'). To detect ELR1, RT-PCR and primer ELR1.fw (5'-CCAAGGCTGATGCCCTGAGC-3') and primer ELR1.rv (5'-GGCCTGGCAG CTCTCGGCAG-3') were used to verify equine cDNA. PCRs were run for 30 cycles of 94°C for 30 s, 1 min at 60°C, and 72°C for 2 min.

Quantitative real-time PCR analyses of eqA3 mRNA. Total RNA was isolated from equine PBMCs and macrophages using an RNeasy minikit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Isolated RNA was reverse transcribed with QuantiTect Reverse Transcription (Qiagen, Hilden, Germany). The eqA3 fragments were amplified from cDNA and quantified using SYBR green PCR Master Mix (Applied Biosystems, Warrington, United Kingdom) with an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) and A3-specific primers as follows (fw indicates forward and rv indicates reverse): qPCR-A3Ccd.fw (5'-ACCATCATGGAAGGC ACTTCATTCC-3') and qPCR-A3Ccd.rv (5'-TGCAGCTTCTCCAATGGGTT TTTGA-3') for A3Z2c-Z2d; qPCReq3Ce.fw (5'-AGTGGAAGGAAGGTTGA GCGGC-3') and qPCReq3Ce.rv (5'-CGGAACCAATCGAGGAAGCAGA-3') for A3Z2e; and qPCReq3H-2.fw (5'-CCGGGAACGGAAACACACAGCATC-3') and qPCReq3H-2.rv (5'-ACGGAGCAGCTTCAGCTGGTAGCA-3') for A3Z3. After initial incubations at 50°C for 2 min and 95°C for 10 min, 40 cycles of amplification were carried out for 15 s at 95°C, followed by 1 min at 60°C. The amplification product was normalized to that of equine GAPDH.

Sequencing of viral transcripts. CrFK cells (1 x 10^6) were infected with DNase I (Roche, Mannheim, Germany)-treated EIAV-luc (VSV-G pseudotyped) (1,000 pg of reverse transcriptase) using pONY8.1luc, pONY3.1, and pMD-G produced in 293T cells together with eqA3s or pcDNA3.1(). At 10 h postinfection, cells were washed with phosphate-buffered saline (PBS), and DNA was isolated using a DNeasy DNA isolation kit (Qiagen, Hilden, Germany). An 880-bp fragment of the luciferase gene was amplified using *Taq* DNA polymerase (Fermentas, St. Leon-Rot, Germany) and the primers 5 luc3@812nt (5 -GATA TGTGGATTTCGAGTCGTC-3) and Eluc.rv (5 -AGGCGGCCGCTTTACAC GGCGATCTTT-3). Each of 30 cycles was run at 95°C for 30 s, 50°C for 1 min, and 72°C for 2 min. For better selective amplification of hypermutated viral genomes, the PCR denaturation temperature was lowered to 88°C (30 cycles of 1 min at 88°C, 1 min at 50°C, and 2 min at 72°C). The PCR products were cloned into pJet1.2/blunt vector (Fermentas, St. Leon-Rot, Germany) and sequenced. The nucleotide sequences of at least 10 independent clones were analyzed.

Immunoblot analysis. Cells were cotransfected with plasmids for viral vectors of EIAV and A3 expression plasmids, and lysates were prepared 2 days later. Cell lysates were prepared by removing the medium from transfected cells, washing them with PBS, and lysing them in radioimmunoprecipitation assay buffer (25 mM Tris · HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate). Virions were pelleted by centrifugation of filtered (0.45-μm-pore size) culture supernatant through a 20% sucrose cushion at 35,000 rpm in an SW40Ti rotor for 1.5 h and resuspended with radioimmunoprecipitation assay buffer. Protein concentration in the lysates was quantitated using Bradford reagent (Bio-Rad, Munich, Germany). Lysates containing 20 μg of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride filters. Filters were probed with mouse anti-HA antibody (1:10,000 dilution; MMS-101P; Covance, Mu¨nster, Germany), mouse anti-V5 antibody (1:5,000 dilution; MCA1360; ABDserotec, Düsseldorf, Germany), or a mixture of both or with mouse anti-α-tubulin antibody (1:4,000 dilution; clone B5-1-2; Sigma-Aldrich, Taufkirchen, Germany) or mouse anti-VSV-G antibody (1:10,000 dilution; clone P5D4, Sigma-Aldrich, Taufkirchen, Germany), followed by horseradish peroxidase-conjugated rabbit anti-mouse antibody (α-mouse immunoglobulin G-horseradish peroxidase; GE Healthcare, Munich, Germany), and developed with ECL chemiluminescence reagents (GE Healthcare, Munich, Germany).

Immunofluorescence. HeLa cells grown on polystyrene coverslips (Thermo Fisher Scientific, Langenselbold, Germany) were transfected with A3 expression plasmids by applying FuGENE (Roche Applied Science, Mannheim, Germany) transfection reagent (DNA-FuGENE ratio of 1:3). At 1 day posttransfection, cells were fixed in 4% paraformaldehyde in PBS for 30 min, permeabilized in 0.1% Triton X-100 in PBS for 45 min, incubated in blocking solution (10% donkey antiserum [Sigma-Aldrich, Taufkirchen, Germany] in PBS) for 1 h, and treated with anti-HA antibody (MMS-101P; Covance, Mu¨nster, Germany) and anti-nucleolin (ab22758; Abcam, Cambridge, United Kingdom) in a 1:1,000 dilution in blocking solution for 1 h. Donkey anti-mouse Alexa Fluor 488 (Invitrogen, Karlsruhe, Germany) and anti-rabbit Alexa Fluor 594 were used as secondary antibodies in a 1:300 dilution in blocking solution for 1 h. Finally, nuclei were stained using DAPI (4 ,6 -diamidino-2-phenylindole; 1:1000 in PBS) (Millipore, Billerica) for 5 min. The images were captured by using a 40x objective on a Zeiss LSM 510 Meta laser scanning confocal microscope. Optical sections were acquired from 1-/m-thick layers.

Phylogenetic analysis. All A3 sequences in the databases from mammalian species within Laurasiatheria were retrieved for analysis, as follows: *Sus scrofa*, A3Z2 (NM_001097446); *Bos taurus*, A3Z1 (EU864534), A3Z2 (NM_001077845), and A3Z3 (DQ974646); *Ovis aries*, A3Z1 (EU864543), A3Z2 (EU864535), and A3Z3 (NM_001093784); *Canis lupus*, A3Z3 (XM_538369) and A3Z1 (XM_847690); *Felis catus*, A3Z3 (NM_001112710), A3Z2a (ABW83272), A3Z2b (ABW83273), and A3Z2c (ABW83271); *Lynx lynx*, A3Z3 (EU007553) and A3Z2 isolate 1 (EU007546); *Puma concolor*, A3Z2 (EU007545); *Panthera pardus*, A3Z3 (EU007551) and A3Z2 (ABA62303); *Panthera leo*, A3Z3 (U007549), A3Z2 isolate 7 (EU007543), and A3Z2 isolate 2 (EU007544); *Panthera tigris*, A3Z3 (EU007550), A3Z2 (AAY99623), A3Z2 isolate 2 (EU016361), and A3Z2 isolate 3 (EU016362). The sequences were aligned at the amino acid level with MUSCLE (http://www.drive5.com/muscle/) (15, 16), visualized for manual correction with Se-Al (freely distributed by Andrew Rambaut at http://tree.bio.ed.ac.uk/software/seal/), and back-translated to the nucleotide level using PAL2NAL (http://www.bork.embl.de/pal2nal) (88). Maximum-likelihood (ML) phylogenetic analysis was performed with the program RAxML HPC (randomized accelerated maximum likelihood for high performance computing) (84, 85) using the GTR 4 model of evolution and the CAT approximation of rate heterogeneity (83), introducing three partitions that corresponded to each of the codon positions. Four independent runs of 1,000 bootstraps were performed, and for each the final tree topology was optimized without resorting to the CAT approximation. The best of the four final trees was chosen (ln 8697.653751; respective optimized values for the alpha parameter of the gamma function for each codon position were 1.312, 1.015, and 4.327), but there were no significant differences among the corresponding likelihood values using an SH (for Shimodaira and Hasegawa) test (80). Bootstrap support values for the nodes were obtained from the combined four sets of 1,000 trees. A pairwise distance matrix was also generated under the ML framework. Bayesian phylogenetic analysis was performed with BEAST, version 1.4.7 (http://beast.bio.ed.ac.uk) (14), with the GTR+Γ4 model of evolution and for both strict clock and uncorrelated log normal relaxed clock, introducing three partitions that corresponded to each of the codon positions, and unlinking parameters across codon positions. Two independent chains of 50 million steps were calculated, writing every 1,000 steps, and analyzed with a burn-in of 10 million steps. Compatibility of both chains was assessed by calculating the corresponding Bayes factor (86), and both chains were combined into one. The combined chains from the relaxed and the strict clock were also compared by calculating the Bayes factor on the corresponding likelihoods. There was only marginally better support for the relaxed clock calculations (log Bayes factor, 2.1). The Bayesian maximum clade credibility tree was built based on the relaxed clock combined chain, and its likelihood was calculated under ML framework and compared with the best RAxML tree. The Bayesian tree was worse than the best ML tree, albeit not significantly worse in an SH test (ln 8713.565178; score difference between the log ML values for the trees, 15.911427; standard deviation of the score difference after nonparametric bootstrap, 11.028095) (80). The Bayesian posterior probabilities were therefore calculated by projecting the trees from the combined Bayesian chain onto the best ML tree. The reference cladogram for the animal species included in the taxon sample was pruned out from a mammalian tree constructed after the supertree methodology (3), using TREEPRUNER (freely distributed by Olaf Bininda-Emonds at http://www.uni-oldenburg.de/molekularesystematik/33997.html).

Nucleotide sequence accession numbers. The sequences reported here have been deposited in the GenBank database (accession number): eqA3Z1b (FJ532287), eqA3Z2a-Z2b (FJ527822), eqA3Z2c-Z2d (FJ527823), eqA3Z2e (FJ532288), eqA3Z3 (FJ532289), and eqA3Z3v1 (FJ532286).

RESULTS

The evolution of eqA3 implies several gene duplications. In the genome sequence from the horse whole-genome assembly, released by the Broad Institute of MIT/Harvard (NCBI GenBank accession no. NW_001799702), we identified six predicted A3 genes: A3Z1a, -Z1b, -Z2a-Z2b, -Z2c-Z2d, -Z2e, and -Z3. The different A3 genes in the horse genome span ca. 0.25 Mb of the chromosome 28, and closely related genes are clearly organized in tandem repeats (Fig. 1). The phylogenetic relationships of the A3 genes within the placentalia superorder of Laurasiatheria are depicted in Fig. S1 in the supplemental material. Phylogenies reconstructed with ML and with Bayesian methodologies were mainly congruent, with the exception of some ill-defined nodes. Thus, three main clusters can be observed, corresponding to the A3Z1, -Z2, and -Z3 proteins.

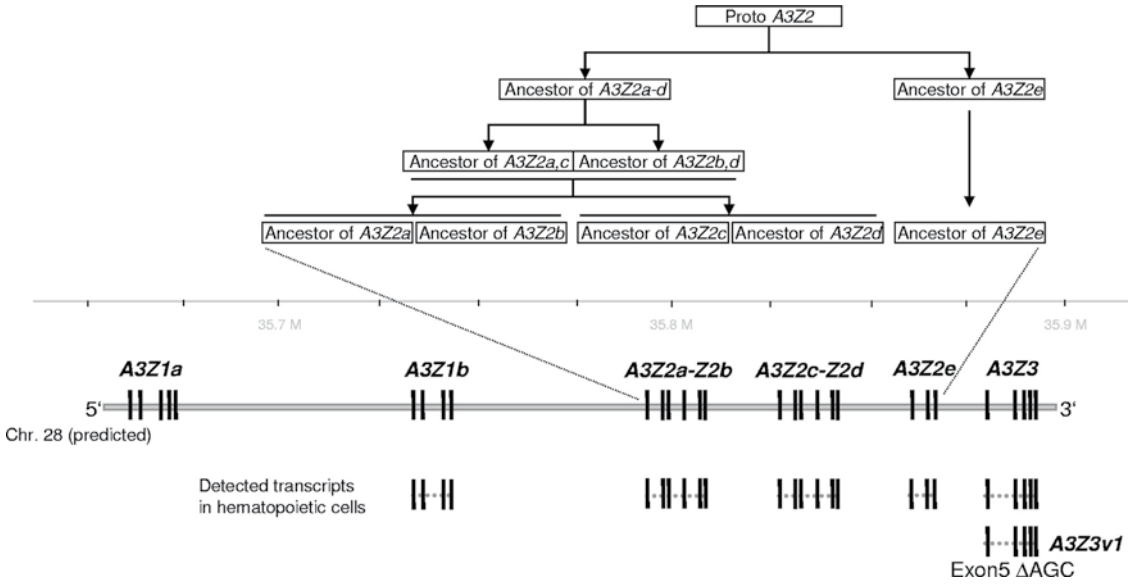

FIG. 1. Identification of A3 coding regions in the genome of *Equus caballus*. Representation of the eqA3 genomic region showing the exon/intron organization of the A3 genes. Transcripts with exons (rectangles) and spliced-out introns (dotted lines) are indicated. Please note that the two eqA3Z3 cDNAs differ in a lack of three nucleotides (AGC) at the 3' end of the A3Z3v1 coding region. The recent study of Bogerd et al. used a different nomenclature for the eqA3 genes: A3A1 for A3Z1a, A3A2 for A3Z1b, A3F1 for A3Z2a-Z2b, A3F2 for A3Z2c-Z2d, A3C for A3Z2e, and A3H for A3Z3 (6). Chr, chromosome.

Each of these clusters reflects grossly the topology of the corresponding species and encompasses genes from different mammalian orders within Laurasiatheria (see Fig. S1 in the supplemental material). Finally, the phylogenetic relationships among eqA3Z2s suggest that the five copies forming the present three genes have appeared after three recent duplication events (Fig. 1).

Identification of A3 RNAs in equine cells. To functionally characterize eqA3s, total RNAs from activated equine PBMCs and equine spleen cells were tested by a combination of RT-PCR and techniques used in 5'/3' rapid amplification of cDNA ends (Fig. 2A). From these sources, we cloned six equine cDNAs (eqA3Z1b, -Z2a-Z2b, -Z2c-Z2d, -Z2e, -Z3, and -Z3v1). The two eqA3Z3 cDNAs differ in a lack of 3 nucleotides (AGC) at the 3 end of the A3Z3 coding region. RNA of the predicted gene for eqA3Z1a could not be identified in hematopoietic cells (Fig. 2A). It has been shown that the in vivo tropism of EIAV is restricted to cells of the monocyte-macrophage lineage (76, 77). Therefore, equine macrophagederived RNA was examined by RT-PCR using eqA3-specific primers. Using semiquantitative PCR on macrophage total RNA, we could detect A3Z2a-Z2b, -Z2c-Z2d, and -Z2e transcripts while we failed to amplify any A3Z1 and -Z3 transcripts (Fig. 2A). Quantitative real-time PCR showed that macrophagesexpress ~19-fold lower levels of A3Z3 than PBMCs (Fig. 2B).

The nomenclature classification of the eqA3s is based on their phylogenetic relationships (see Fig. S1 in the supplemental material) and zinc (Z)-coordinating domain assignments (37): eqA3Z1b has a 45.4% amino acid identity to human A3A(A3Z1a); eqA3Z2a-Z2b and eqA3Z2c-Z2d have 42.9% and 44.3% amino acid identity to human A3F (A3Z2e-Z2f), respectively; eqA3Z2e a has 45% amino acid identity to human A3C (A3Z2b); and eqA3Z3 shares 40.4% identical amino acids with human A3H (A3Z3) (see Fig. S2 in the supplemental material). We used the cloned eqA3Z1b, -Z2a-Z2b, -Z2c-Z2d, -Z2e, -Z3, and -Z3v1 cDNAs to generate expression plasmids for HA-tagged A3s. To characterize subcellular distribution of A3s, cells expressing eqA3s were analyzed by using an anti-HA antibody in confocal microscopy (Fig. 2C). Immunofluorescence studies of transiently expressed eqA3Z1b in HeLa cells showed a strict nuclear localization, as previously reported also for human A3A (57). The subcellular distribution of eqA3Z2a-Z2b is limited to the cytoplasm, and eqA3Z2c-Z2d is localized in the nucleus. In contrast to human A3C that displays a cytoplasmic and nuclear distribution (57), the eqA3Z2e could be detected only in the cytoplasm. The eqA3Z3 and eqA3Z3v1 and the human A3H are distributed in the cytoplasm and in nuclear bodies (Fig. 2C). Costaining with an antibody against the nucleolar nucleolin protein revealed that eqA3Z3 and human A3H localize in the nucleolus to the nucleoli.

eqA3 proteins restrict EIAV, SIV_{agm}, and AAV-2. To examine the restriction activity of eqA3 proteins, VSV-G pseudotyped EIAV-luc and SIV_{agm}-luc reporter viruses were generated in human 293T cells in the presence of cotransfected eqA3 expression plasmids. Reverse transcriptase-normalized EIAV vector particles were used to transduce feline CrFK cells because they show no Trim5α-dependent postentry restriction of EIAV (72, 97). Infectivity of SIV vectors was analyzed by infection of human HOS cells. At 3 days postinfection the cells

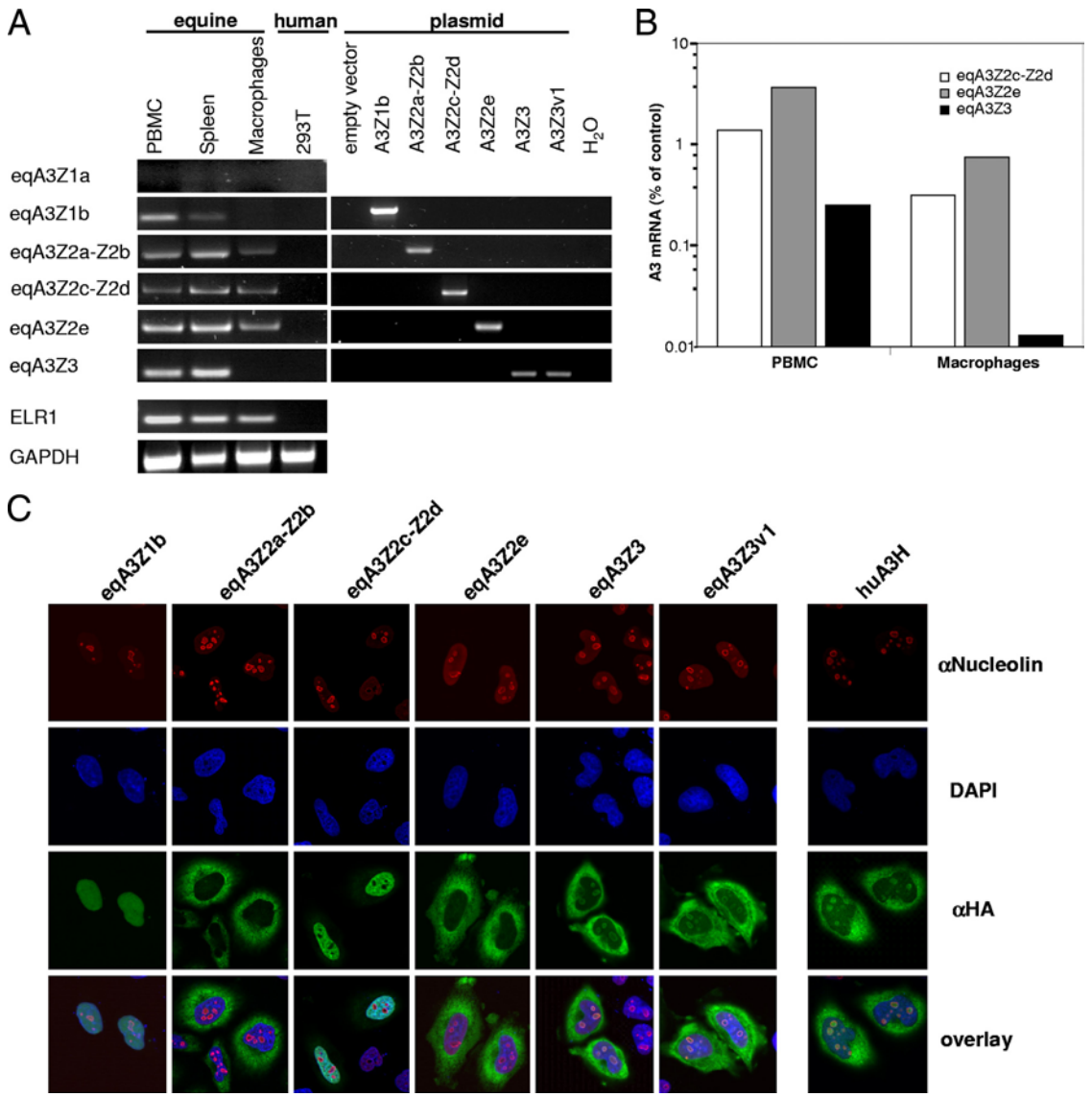

FIG. 2. Differential expression of eqA3s. (A) Analysis of eqA3Z1a, eqA3Z1b, eqA3Z2a-Z2b, eqA3Z2c-Z2d, eqA3Z2e, and eqA3Z3 expression by RT-PCR of total RNA from equine activated PBMCs, spleen cells, and macrophages. Primer specificity in PCR was tested by using eqA3 plasmids as templates. PCRs were performed with specific primers to amplify ELR1 for species control, and GAPDH served as a loading control. (B) eqA3Z3 is expressed at a significantly lower level in macrophages. RNA was extracted from both equine PBMCs and macrophages. Levels of A3 expression were determined by quantitative real-time RT-PCR and expressed relative to the levels of the GAPDH RNA. (C) eqA3s show a different subcellular localization. HeLa cells were transfected with HA-tagged eqA3Z1b, eqA3Z2a-Z2b, eqA3Z2c-Z2d, eqA3Z2e, and eqA3Z3 and with human A3H and A3Z3. To detect A3 (green) immunofluorescence, staining was performed with an anti-HA antibody. Nuclei (blue) were visualized by DAPI staining. Nucleolar nucleolin protein (red) was detected with anti-nucleolin antibody. α, anti; hu, human.

were lysed and used to quantify intracellular luciferase activity. In addition, we tested the inhibitory capacity of the eqA3s on the transduction of AAV-2-lacZ reporter vectors. We found that the eqA3 expression plasmids produced functional active antiviral proteins and that most showed a distinct specificity against different viruses (Fig. 3A to C). All six eqA3 proteins were detectable in purified EIAV particles by immunoblotting (Fig. 3D), suggesting that no viral factor induced their exclusion from EIAV virions. eqA3Z3 restricted any tested viruses: EIAV infectivity was reduced by a factor 60, SIV was reduced by factor 13, and AAV-2 was fivefold inhibited (Fig. 3A to C). The eqA3Z1b and -Z2e showed only moderate activity on EIAV and reduced the infectivity by approximately threefold each (Fig. 3A). Similarly, eqA3Z1b was weakly active against SIV. In contrast, SIV_{agm} infectivity was inhibited 70-fold by eqA3Z2e, and eqA3Z1b reduced the infectivity of AAV-2 by

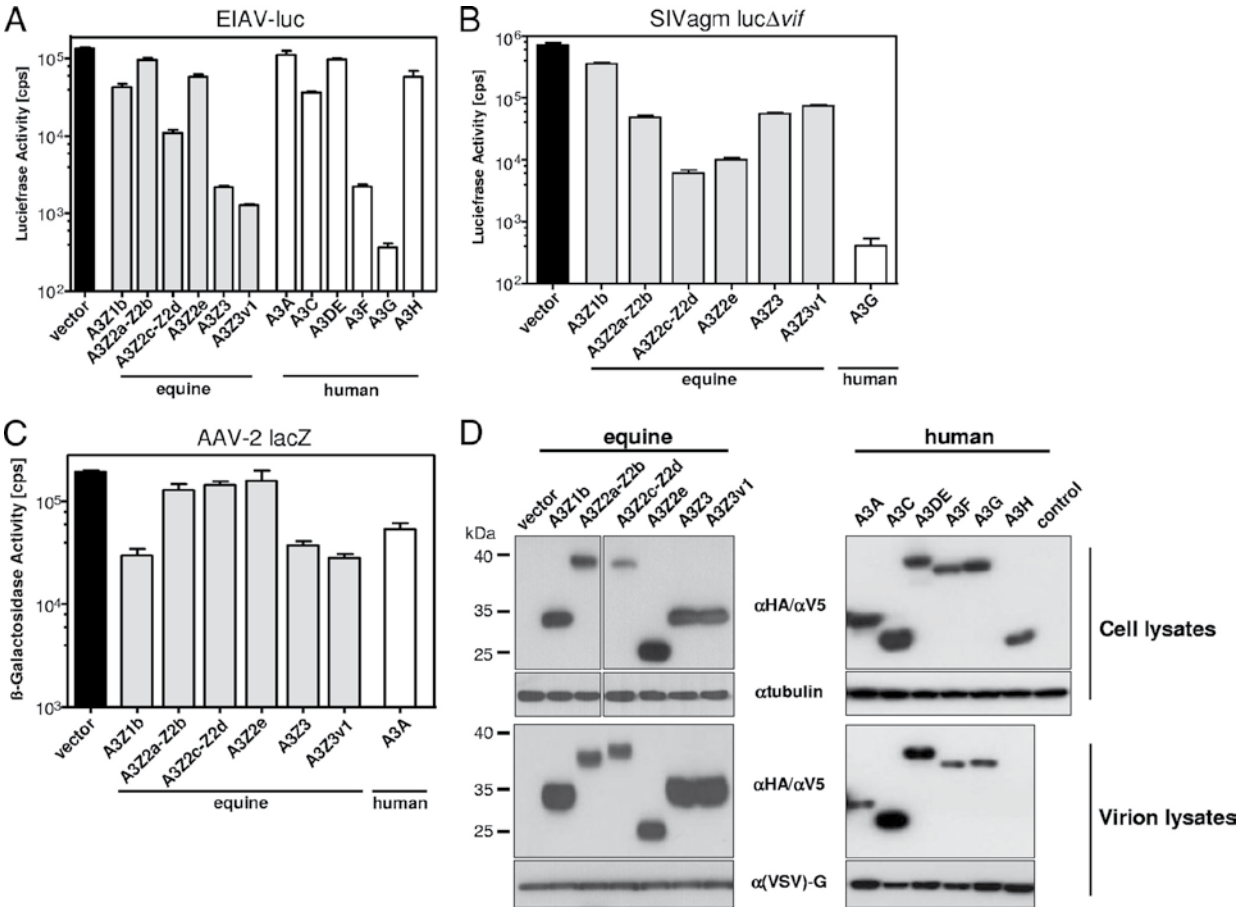

FIG. 3. eqA3 proteins inhibit the infectivity of different viruses. (A) EIAV-luc (VSV-G pseudotyped) was produced in 293T cells in the presence or absence of the indicated equine and human A3s. Infectivity of the viruses was determined by quantification of luciferase activity in CrFK cells with equal amounts of viruses at 3 days postinfection. (B) SIVagm *vif* viruses were produced in the presence or absence of the indicated A3 proteins. Infectivity of normalized amounts of viruses was determined by quantification of luciferase activity at 3 days postinfection. (C) AVV-2 viruses were generated by transfecting 293 cells in the presence or absence of the indicated A3 proteins. Antiviral activity of A3 proteins was determined by quantification of ß-galactosidase expression at 3 days postinfection. (D) Equine and human A3 proteins are packaged in EIAV virions. Virions were generated by cotransfection of 293T cells with EIAV-luc and HA- or V5-tagged equine and human A3 expression plasmids or pcDNA3.1 (vector). A3 expression in transfected cells was detected by immunoblotting using a mixture of anti-HA and anti-V5 antibodies. Cell lysates were also analyzed for equal amounts of total proteins by using anti-tubulin antibody. Packaged A3s in virions (normalized by RT activity) were detected by probing with anti-HA and anti-V5 antibody together on a parallel immunoblot. Immunoblots of virions were also probed with anti-VSV-G antibody as a control for the equal amounts of analyzed virions. cps, counts per second; a, anti.

10-fold (Fig. 3B and C). eqA3Z2c-Z2d restricted both EIAV and SIV by a factor of 12 and a factor of 120, respectively (Fig. 3A and B). Our results on the activity of A3Z2c-Z2d are in contrast to the report of Bogerd et al. (6). Comparing the sequence of A3Z2c-Z2d of Bogerd et al. (6), designated A3F2, with our A3Z2c-Z2d expression plasmid revealed four different amino acids (V40G, P48V, S339I, and K354N) (see Fig. S3 in the supplemental material). We introduced these amino acid changes in our construct and tested A3F2 for its antiviral activity (Fig. 4A). Both A3 proteins showed similar protein expression levels (Fig. 4B) and identical subcellular distributions (Fig. 2C) (compare to reference 6), but A3F2 reduced the infectivity of EIAV only twofold (Fig. 4A), confirming the results of Bogerd et al. (6). We conclude that natural sequence variants of some eqA3s can have a strong impact on their activity against EIAV.

To address the question whether eqA3 proteins show a cytidine deaminase activity, EIAV vector cDNAs were analyzed by PCR. We recovered an ~880-bp fragment of the luciferase gene from infected cells, 10 h postinfection. The rate of the detected G→A mutations in viral RT products was very low. The G→A mutation rate was equal to or less than 0.07% of all nucleotides for eqA3Z2a-Z2b, -Z2c-Z2d, and -Z3, and no G→A mutations were detectable for eqA3Z1a and -Z2e (Fig. 5A, left panel). Based on the unsatisfactory results for deamination activity of eqA3 proteins, we modulated the PCR con-

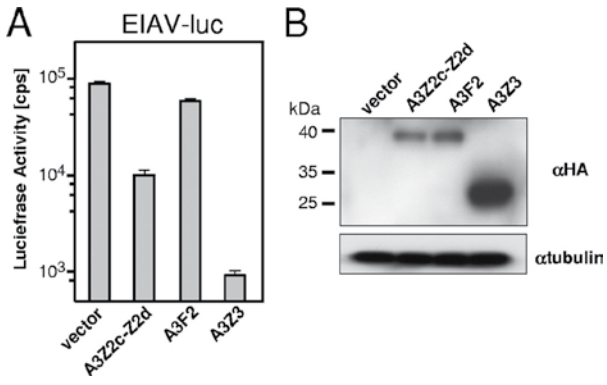

FIG. 4. Double domain eqA3Z2c-Z2d protein variants differ in their antiviral activity. (A) Single-cycle infectivity assays were performed for EIAV in presence or absence of A3Z2c-Z2d and the variant A3F2 differing in four amino acids. Infectivity of equal amount of viruses was determined by quantification of luciferase activity. (B) Immunoblot analysis of the indicated eqA3 proteins using an HA tag-specific antibody. α, anti; cps, counts per second.

ditions to selectively amplify AT-rich DNA generated by G→A hypermutations, as previously described for a three-dimensional PCR method (87). Indeed, the rate of G→A mutations detected by PCR using a denaturation temperature of 88°C was ~7% of all nucleotides for eqA3Z3 and ~3% for A3Z2c-Z2d (Fig. 5A, right panel; see also Table S1 in the supplemental material). In experiments with eqA3Z1b, -Z2a-Z2b, and -Z2e, a low rate of 0.2 to 0.5% G→A mutations was now detected, corresponding to their very weak antiviral activity against EIAV. Under this PCR condition, no amplification product was detectable in experiments omitting eqA3. The editing context of the G→A exchanges in EIAV genomes displays a dinucleotide preference of GG→AG and GA→AA (Fig. 5B). In conclusion, eqA3s harbor the intrinsic activity to deaminate cytidines and can deaminate EIAV genomes in at least a subpopulation of the transducing particles under experimental conditions.

The dUTPase and S2 genes do not affect the susceptibility of EIAV to eqA3s. EIAV encodes two genes, dUTPase (DU) and S2, that are absent in the genome of HIV-1. To investigate the possibility that DU or S2 counteracts the antiviral activity of eqA3s, EIAV DU and S2 mutation constructs were generated. Since there were no antibodies against DU and S2 available, no further biochemical studies on these proteins were performed. Two EIAV-packaging constructs were tested in which either the *DU* gene was inactivated by an in-frame deletion of 270 bp (EIAVΔ*DU*) identical to the method of Threadgill et al. (90) or the *S2* gene was inactivated by a deletion of 52 bp including the ATG start codon of S2 (EIAVΔS2), as described previously (55). We produced EIAV vectors of wt, ΔDU, or ΔS2 with and without expression plasmids for eqA3s and measured the particle infectivity on feline CrFK cells and on the equine macrophage-like cell line EML-3C. The results shown in Fig. 6 demonstrate that deletion of DU or S2 did not change the antiviral activities of the eqA3Z2a-Z2b or -Z2e that are inactive against wt EIAV. Moreover, wt, ΔDU, and ΔS2 vectors were equally inhibited by eqA3Z1b, -Z2c-Z2d, and -Z3. Thus, we conclude that the resistance of EIAV to eqA3Z2a-Z2b and -Z2e is not based on the function of the dUTPase or the S2 protein and that DU and S2 are not required for transduction of equine macrophage-like cells.

EIAV restriction by human and nonprimate A3 proteins. Previous studies investigated exclusively whether human A3A and human A3G are antiviral active against EIAV (6, 48). To determine if other human and nonprimate A3 proteins exert an inhibitory effect on the infectivity of EIAV, we cotransfected 293T cells with the EIAV-luc reporter vector together with expression plasmids encoding human A3A, -B, -C, -DE, -F, -G, or -H; feline A3Z2a, -Z2b, -Z2c, -Z3, or Z2b-Z3; canine A3Z1, or -Z3; porcine A3Z2-Z3; or muA3Z2-Z3. In experiments using the human A3B expression plasmid, no RT activity after cotransfection with the EIAV vector system was detectable in the cell supernatant. Since the expression of the EIAV vectors is driven by the cytomegalovirus promoter, this result would agree with a previous study of Zhang et al., where the authors concluded that human A3B is able to inhibit transcription of simian virus 40 and cytomegalovirus promoters (99). Again, CrFK cells were infected with vector stocks normalized for RT, and 3 days postinfection the cells were used to quantify intracellular luciferase activity. As shown in Fig. 3A and 7, human A3F and -G, muA3Z2-Z3, and porcine A3Z2-Z3 proteins were found to exert a significant inhibitory effect on the infectivity of EIAV. Specifically, human A3G and porcine A3Z2-Z3 reduced the infectivity of EIAV 90-fold and 145-fold, respectively. The other tested human and nonprimate A3s showed no effect or a very weak inhibitory effect against EIAV. Human A3s were packaged in EIAV independent of their antiviral activity, with the notable exception of human A3H (Fig. 3D).

A single amino acid mutation in the active site of eqA3Z3 results in loss of antiviral activity. Because of the difficulties in detecting eqA3-derived cytidine deamination in EIAV genomes, we studied the relevance of the zinc coordination domain for the antiviral activity. It has been reported that cytidine deamination is largely dispensable for the inhibition HIV-1 Δ*vif* by human A3F and A3G (4, 30, 46, 61). But controversially, several groups have reported a significant drop in inhibition observed when active-site mutants of human A3G were analyzed (8, 9, 22, 23, 56, 75). To address this question for EIAV, we analyzed four previously described human A3F and -G mutants called h3FAS1, h3FAS2, h3FAS1/2, and A3G-E259Q (7, 23). Additionally, we generated four eqA3Z3 mutants with related changes at the important amino acid positions of the Zn^{2+} coordinating site. Human A3F and -G contain two Zn^{+} coordinating sites; only the CT Zn^{2+} coordinating sites are enzymatically active while the amino-terminal Zn^{2+} coordinating sites have been proposed to play a role in the specific packaging of these two A3s into HIV-1 Δ*vif* particles (23, 60). In A3G-E259Q, a glutamic acid residue at position 259 has been mutated to glutamine, which results in loss of the cytidine deaminase activity of A3G (7). The catalytic glutamic acid residues of the amino-terminal cytidine deaminase domain (CDD) in the A3F mutant h3FAS1 and of the CT CDD in h3FAS2 are changed to alanines, and h3FAS1/2 carries both point mutations (23). For eqA3Z3, in the single CDD the mutations H84R, E86Q, C115S, and C118S were gener--

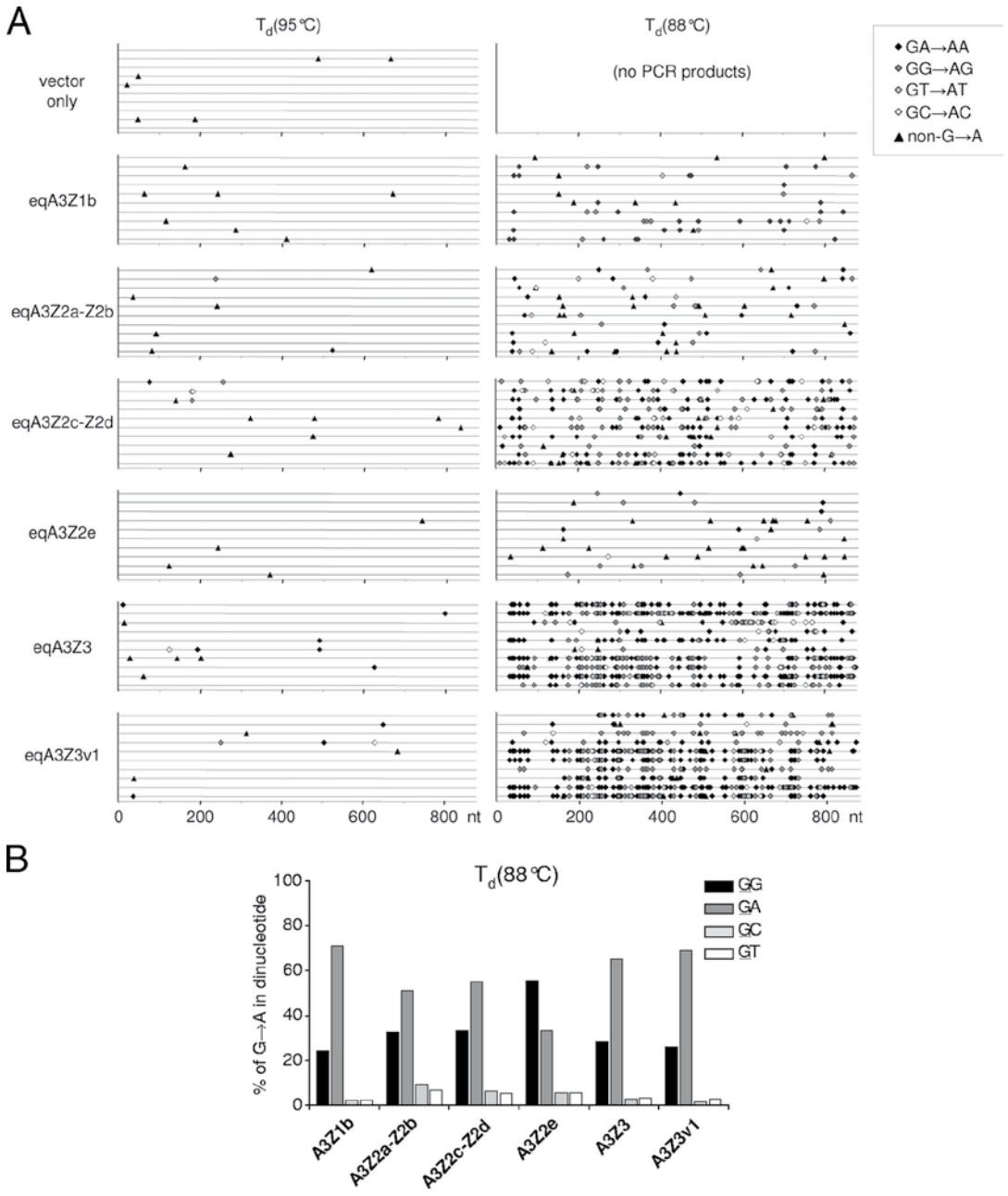

FIG. 5. eqA3 triggers G-to-A hypermutation in a subpopulation of the EIAV-luc genomes. (A) EIAV vector sequences (880 nucleotides [nt] of the luciferase gene) amplified by PCR at 10 h postinfection from CrFK cells transduced by EIAV generated in the presence or absence of eqA3Z1b, eqA3Z2a-Z2b, eqA3Z2c-Z2d, eqA3Z2e, eqA3Z3, and eqA3Z3v1. The mutations of 10 individual clones of each group are shown. Each mutation is indicated and coded with respect to the nucleotide mutation. PCR was done at a denaturation temperature (T_d) of either 95°C or of 88°C. (B) Comparison of the dinucleotide sequence context of G (underlined) →A mutations in the positive-strand DNA of EIAV-luc derived from eqA3-expressing 293T cells.

ated, resulting in eqA3Z3-H84R, -H86Q, -C115S, and -C118S, respectively. Analyses of the effect of the A3F and A3G mutants on EIAV infectivity showed that the A3 CT mutants lost all or most of the antiviral activity: h3FAS2 and hA3FAS1/2 reduced the infectivity only two- to threefold, and A3G-E259Q showed no inhibitory effect; wt A3F and the mutant h3FAS1 inhibited EIAV 10- and 15-fold, respectively (Fig. 8A). The mutant h3FAS1 was slightly more active than the wt human A3F, an effect also observed by Hakata et al. in connection with HIV-1Δ*vif* (23). The protein A3G-E259Q showed a reduced packaging, while all other active-site mutants were found in EIAV particles at amounts similar to wt proteins (Fig. 8B). The eqA3Z3 mutants showed weak antiviral activity and inhibited EIAV only twofold (Fig. 8A). We conclude that the

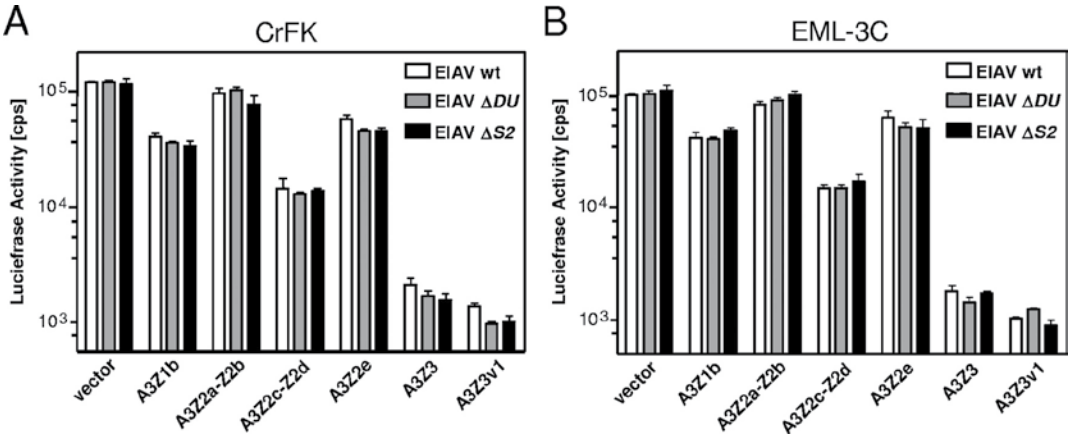

FIG. 6. Inhibition of EIAV by eqA3s is independent from dUTPase and S2. wt, ΔDU, and ΔS2 EIAV viruses were produced in the presence or absence of the indicated HA-tagged A3 expression vectors. Relative infectivity of equal amounts of the reporter viruses was determined by quantification of luciferase activity in CrFK cells (A) or EML-3C macrophage-like cells (B) at 3 days postinfection. cps, counts per second.

CT CDDs of human A3F and -G also play an essential role in the inhibition of EIAV and that the CDD of eqA3Z3 is important even though CDD mutants of eqA3Z3 remain partially active against EIAV.

DISCUSSION

The evolution of A3 genes tells us a story of gene duplication and specialization throughout mammalian history. Three main clades can be distinguished within the A3 genes, namely A3Z1, A3Z2, and A3Z3. The distribution supports the hypothesis of an ancient origin of the A3 genes in the proto-mammalian genome, followed by early duplication events before the appearance of the main clades within Mammalia (38, 58). In this sense, the chromosomal arrangement of the A3 genes in the horse genome is paradigmatic: two copies of the A3Z1 genes, followed by five copies in tandem of the A3Z2 genes, and a single copy of the A3Z3 gene. Regarding the two eqA3Z1 genes, a homologous canine A3Z1 gene exists as a single gene (58). This suggests that the extant A3Z1a and A3Z1b genes in the horse genome appeared via duplication after the last common ancestor of Carnivora and Perissodactyla, which could be dated some 62.5 million years ago (2). With respect to origins of the five eqA3Z2 genes, they all also share a common ancestor that is more recent than the last common ancestor of Perissodactyla and Carnivora and should therefore be considered inparalogs compared with the rest of the tree terminal taxa (81). In the Bayesian relaxed clock results, there are no significant differences between the heights of the nodes that define the last common ancestor of the eqA3Z1 genes (median, 0.0633; 95% confidence interval, 0.033 to 0.1034 substitutions per site) and the eqA3Z2 genes (median, 0.0742; 95% confidence interval, 0.0525 to 0.105 substitutions per site). This concordance can be interpreted to mean that both expansions, A3Z1 and A3Z2, have occurred close in time, maybe even in two simultaneous duplication events. This event(s) would have generated, on the one hand, the ancestors of the present day eqA3Z1a and eqA3Z1b and, on the other hand, the ancestor of present day eqA3Z2a-d and of eqA3Z2e (Fig. 1). After the duplication of the ancestor A3Z2a-d gene, which generated the ancestor A3Z2a,c and A3Z2b,d genes, these later genes fused and formed after an additional duplication the present A3Z2a-Z2b and A3Z2c-Z2d double-domain A3 genes. While the ancestor A3Z2a-d was involved in several genetic rearrangements, the A3Z2e gene was preserved. Further research will be necessary to determine whether the additional duplications of the A3Z1 genes and of the A3Z2 genes in horse are shared also by other species within the genus *Equus* (such as zebra or donkey) or by other genera within the order Perissodactyla (such as Rhinocerotidae or Tapiridae). Recently, read-through transcripts of A3Z2 and A3Z3 genes have been identified in cats and pigs (38, 58). We could not find such a transcript in our equine cDNA samples. But very interestingly, an equine

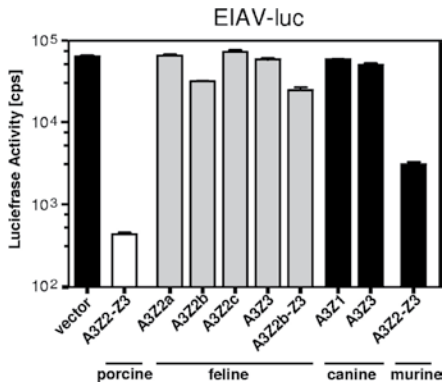

FIG. 7. Inhibition of EIAV by nonprimate A3 proteins. EIAV luciferase reporter viruses were produced in 293T cells in the presence or absence of the indicated A3s. Infectivity of the viruses was determined by quantification of luciferase activity in CrFK cells infected with equal amounts of virions at 3 days postinfection. cps, counts per second.

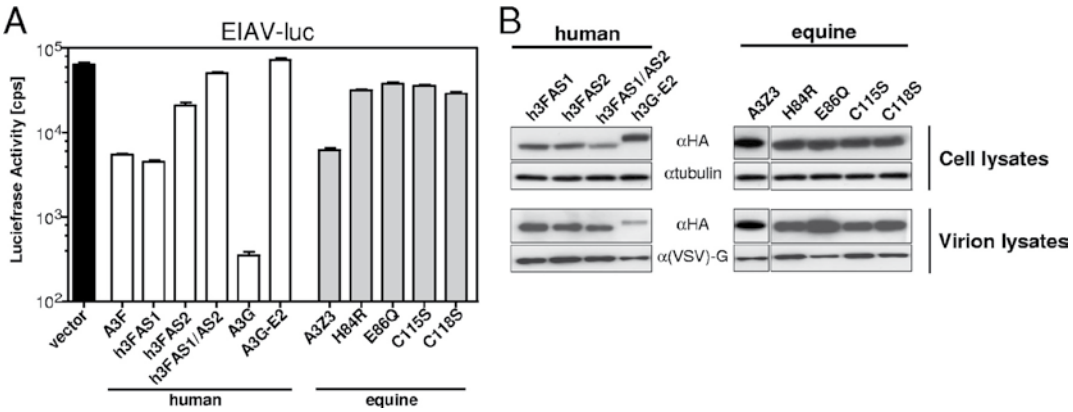

FIG. 8. Analysis of human A3F and A3G and eqA3Z3 active-site mutants for antiviral activity. (A) Different active-site mutants of human A3F and A3G and eqA3Z3 (A3Z3) were tested for antiviral activity against EIAV-luc produced in 293T cells by cotransfection with wt or mutant A3 expression plasmids. Transfection with pcDNA3.1 (vector) instead of the A3 expression plasmid was included as a control. CrFK target cells were infected with virus-containing supernatants normalized for RT, and luciferase was measured at 3 days postinfection. (B) Virions were generated by cotransfection of 293T cells with EIAV-luc and mutant HA-tagged eqA3 and human A3 expression plasmids or pcDNA3.1 (vector). A3 expression in transfected cells was detected by immunoblotting using anti-HA antibodies. Cell lysates were also analyzed for equal amounts of total proteins by using anti-tubulin antibody. Packaged A3s in virions normalized by RT activity were detected by probing with anti-HA antibody. Immunoblots of virions were also probed with anti-VSV-G antibody as a control for equal particle loading. α, anti.

read-through transcript is deposited in the GenBank database (RNA molecule accession number XM_001501833). This at least implies that some individual horses do express an additional A3.

Within the placentalia superorder of Laurasiatheria, A3Z2 genes have also undergone several duplication rounds in cats (58). Remarkably, the A3Z3 gene was never amplified and has been preserved as a single gene in Felidae and in Equidae. A similar situation is found within the superorder of Euarchontoglires in humans/primates, where after the amplification of A3Z2 and A3Z1, the duplicated genes fused and formed double-domain A3 genes (human A3B, -DE, -F, and -G) (37, 58). Amplified A3Z3 genes have not been found, and in contrast to amplification of A3Z1 and A3Z2 genes, it seems to be an evolutionary disadvantage. Consistent with this hypothesis, it was recently shown that nonfunctional human A3Z3 alleles are highly prevalent and have been actively selected for (24, 62). The discovery of the cellular antiviral cytidine deaminase A3G in search for the Vif cofactor explained many in vitro observations regarding the replication of HIV-1 and HIV-1 Δ*vif* in specific human cells (5, 49, 78, 91, 100). It also contributed to explain the narrow host range of HIV-1 that shows spreading replication only in humans and chimpanzees (49, 59). Such defined interactions of Vif proteins with cognate A3 proteins had been shown so far only for HIV, SIV, and *Feline immunodeficiency virus* (49, 58, 74). But the discovery of the Vif-A3 interaction also highlighted the conundrum of "simple" mammalian retroviruses like Mo-MLV and *Mouse mammary tumor virus* and their survival without accessory genes like the *vif* gene (45,64–66). If simple retroviruses do get along without encoding proteins that actively counteract the impact of antiviral A3s, it is unclear why lentiviruses have evolved or retained the *vif* gene. A Vif-deficient lentivirus like EIAV might therefore be a possible evolutionary link to understand the biological relevance of Vif and to further investigate the question whether a virus is targeted by its species-own A3s (70). Equine PBMCs express at least five different types of A3 genes. EIAV was mostly sensitive to eqA3Z3 and eqA3Z2c-Z2d and was only weakly inhibited by eqA3Z1b, eqA3Z2a-Z2b, and -Z2e. However in macrophages, the natural target cells of EIAV, anti-EIAV A3s (A3Z2c-Z2d and A3Z3) are expressed at low levels or barely expressed. Some reports have suggested a requirement for the S2 gene for high virus loads in vivo and disease induction (19, 42). Our finding that EIAV S2 does not affect susceptibility to eqA3s is consistent with studies by Li et al. (43), who have recently shown that S2 is not required for viral replication in vitro in several equine cell lines and macrophages. In contrast, the dUTPase of EIAV is necessary for viral replication in macrophages (17). Our data clearly show that EIAV dUTPase does not protect against eqA3s. How does EIAV survive and replicate without any A3-counteracting viral function? We propose that EIAV may use multiple strategies to interact with its species-own A3s: it tolerates packaging of eqA3s that are not active against EIAV (A3Z1b, A3Z2a-Z2b, and A3Z2e) and avoids the active antiviral eqA3Z3 by an evolutionary adaption of its cellular tropism to macrophages that showed very low expression levels of A3Z3. The A3Z2c-Z2d was also active against EIAV and found to be expressed at low levels in macrophages. Interestingly, the sequence of the eqA3Z2c-Z2d of our horse samples compared with the sequences of Bogerd et al. (6) differed in four amino acids. This genetic variation in A3Z2c-Z2ds influenced its anti-EIAV activity significantly (Fig. 4A). Future experimental investigations and field studies are required to support our hypothesis that the presence of these or other A3 single nucleotide polymorphisms and the expression of A3Z2-Z3 read-through transcripts modulate the susceptibility of horses to EIAV infection and disease induction.

The evolutionary history of the A3 genes shows a clear tendency toward the growth of the antiviral protein armory. This increase proceeds through gene duplication and possibly also gene fusion and alternative read-through mechanisms. Since gene fusion is more common than gene fission (36), it is tempting to speculate that the read-through mechanism could act as a preadaptation, facilitating—even mechanistically—the gene fusion event. The diversification of genes opens the door to neofunctionalization (29) and/or subfunctionalization (47). Under the "duplication-degeneration-complementation" model, mechanisms that tend to preserve the duplicated genes act by partitioning the original functions in the ancestral gene between the sister genes rather than generating new functions (21). We have shown here that present-day eqA3 proteins appeared after recent gene duplication events, have different cellular localizations, and display different activities against distinct viruses, and this is also true for other species (58). In this sense, the different eqA3Z1 and eqA3Z2 proteins, the different human A3Z2-Z2 and -Z2-Z1 proteins, the different feline A3Z2 proteins, and even A3Z2-Z3 read-through transcription products (38, 58) might be regarded as specialized antiviral proteins that have evolved, taking over and refining ancestral functions in the original gene. Alternatively, if the evolutionary forces that have independently favored the conservation of the duplicated A3 genes in Equidae, in Felidae, and in primates parallel the pressure that retroviruses pose on their hosts, the maintenance of the duplicated genes in the host genome might reflect an increase in the retroviral diversity affecting specific taxa at specific time points.

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CHAPTER 5

Vif of Feline Immunodeficiency Virus from Domestic Cats Protects against APOBEC3 Restriction Factors from Many Felids

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To get more insight into the role of APOBEC3 (A3) cytidine deaminases in the species-specific restriction of feline immunodeficiency virus (FIV) of the domestic cat, we tested the A3 proteins present in big cats (puma, lion, tiger, and lynx). These A3 proteins were analyzed for expression and sensitivity to the Vif protein of FIV.While A3Z3s and A3Z2-Z3s inhibited Δ*vif* FIV, felid A3Z2s did not show any antiviral activity against Δ*vif* FIV or wild-type (wt) FIV. All felid A3Z3s and A3Z2-Z3s were sensitive to Vif of the domestic cat FIV. Vif also induced depletion of felid A3Z2s. Tiger A3s showed a moderate degree of resistance against the Vif-mediated counter defense. These findings may imply that the A3 restriction system does not play a major role to prevent domestic cat FIV transmission to other *Felidae*. In contrast to the sensitive felid A3s, many nonfelid A3s actively restricted wt FIV replication. To test whether Vif$_{FIV}$ can protect also the distantly related human immunodeficiency virus type 1 (HIV-1), a chimeric HIV-1. Vif$_{FIV}$ was constructed. This HIV-1.Vif$_{FIV}$ was replication competent in nonpermissive feline cells expressing human CD4/CCR5 that did not support the replication of wt HIV-1. We conclude that the replication of HIV-1 in some feline cells is inhibited only by feline A3 restriction factors and the absence of the appropriate receptor or coreceptor.

In the family of *Retroviridae*, the *vif* gene is present in most members of the genus *Lentivirus*. Its absence in *Equine infectious anemia virus* and in endogenous lentiviruses of the order *Lagomorpha* might indicate that *vif* appeared later in lentivirus evolution and strongly emphasizes that lentivirus replication is not strictly dependent on Vif (20, 58). Nevertheless, in the well-studied primate and feline lentiviruses, viruses with a *vif* deletion barely replicate *in vivo* and are not pathogenic (10, 46). Functional studies revealed that the Vif protein of the human immunodeficiency virus type 1 (HIV-1) binds in the virus-producing cells the cellular cytidine deaminases APOBEC3F (A3F) and APOBEC3G (A3G) and induces their polyubiquitination and subsequent degradation by the proteasome (27, 45, 54, 55). In addition, it is reported that Vif of HIV-1 inhibits APOBEC3 (A3) proteins by other, nondegrading mechanisms (for a review, see reference 6).

During viral infections when either no Vif protein is made or when HIV-1 faces A3 proteins normally absent in T cells or macrophages, A3s can be incorporated in the viral particles and often inhibit the next round of infection. Other than the cytidine deaminase activity on single-stranded viral DNA during reverse transcription, human A3F and A3G inhibit Δ*vif* HIV-1 by other means (2, 15, 16, 18, 28, 34). The finding that HIV-1 does not infect species besides *Homo sapiens* and *Pan troglodytes* is based on the species-specific adaptation of Vif to human A3s and other host-virus restriction mechanisms. In support of this model, HIV-1 is inhibited by A3s of rhesus macaques, African green monkeys (AGM), pigs, mouse, cats, and horses (4, 19, 25, 33). In the case of A3G from AGM, a single amino acid at position 128 determines whether Vif of HIV-1 or simian immunodeficiency virus (SIV) of AGM can or cannot counteract its antiviral activity (3, 24, 43, 53).

Similar to the situation of SIVs in primates, specific types of the feline immunodeficiency virus (FIV) can be isolated from many *Felidae* (49). Little is known about whether the diverse FIV isolates have a restricted host range as do HIV-1 and the SIVs (32). The phylogenetic data indicate that most of these FIV types isolated from different felids are monophyletic (summarized in references 39 and 48). However, recent FIV cross-species transmissions have been observed in pumas infected by the FIVs of domestic cats and bobcats, in leopards and tigers infected with the FIVs of lions, and in free-ranging leopard cats infected with FIV strains of domestic cats, indicating that repeated and/or multiple historic FIV cross-species felid-to-felid transmission events can occur in the wild or in captivity (5, 13, 35, 39, 48, 51). With the exception of repeated FIV transmissions from bobcats to pumas, these reports mostly describe singular events supporting that FIV is not frequently cross-species transmitted.

Recently, we characterized the feline *Felis catus* A3 (FcaA3) locus showing that it is less complex than that of primates but significantly more evolved than that of rodents (31). Based on an evolution-based nomenclature that reflects the identity of the zinc (Z) coordinating domain(s) (21), cats encode a triplicate Z2 and a single Z3 A3 gene. In addition, a complex process of readthrough transcription and alternative splicing results in two-domain feline A3Z2-Z3 molecules (31; see also below). These different feline A3 restriction factors display a specific pattern of restriction toward wild-type (wt) feline retroviruses (FIV, *Feline leukemia virus* [FeLV], and *Feline foamy virus* [FFV]) and variants devoid of their counterdefense proteins: while wt FIV and FFV are almost resistant against any of the feline A3s, FeLV is moderately inhibited by FcaA3Z2-Z3s. In contrast, Vif-deficient FIV is restricted by the two-domain FcaA3Z2-Z3 and slightly by the Z3 form, while Bet-deficient

FFV is restricted to variable degrees by the three FcaA3Z2 proteins (22, 31).

In the present study, we sought to determine whether the Vif protein of the domestic cat FIV is counteractive against A3s of four lineages within *Felidae*. Using single-round replicating FIV-reporter viruses, we detected a very broad activity of Vif_{FIV} inhibiting almost all tested A3s of *Felidae* similar to their own species A3s. We further found that vif_{FIV} cloned into HIV to replace the expression of authentic *vif* enables such chimeric HIV-1 to overcome the A3 restriction in feline cells, allowing productive replication in cat cells engineered to express the human receptors for HIV-1.

MATERIALS AND METHODS

Cells and transfections. The human cell lines 293T, HeLa, HOS (ATCC CRL-1543), HOS.CD4.CCR5 (NIBSC ARP078), and the modified feline cell line CrFK.CD4.CCR5 (33) were maintained in Dulbecco high-glucose modified Eagle medium (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). Ficoll-enriched human peripheral blood mononuclear cells (PBMC) were stimulated with 5 µg of phytohemagglutinin (Murex Diagnostics, Ltd., Dartfield, United Kingdom)/ml and incubated with 30 IU of human IL-2 (Proleukin; Chiron, Emeryville, CA)/ml in RPMI 1640 containing 10% FBS and 2 mM Glutamax I (Invitrogen, Karlsruhe, Germany) for 48 h before infection. Plasmid transfections into 293T cells were performed with Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. Transfections were done in 12-well plates seeded with 2.5 x 10^5 cells/well the day before transfection and 2.2 µg of plasmid DNA. The standard transfection experiment contained 0.5 µg of FIV packaging construct, 0.5 µg of FIV-luciferase vector, 0.5 µg of A3 expression plasmid, 0.5 µg of Vif expression plasmid, and 0.2 µg of VSV-G expression plasmid; in some experiments pcDNA3.1(+) (Invitrogen) was used instead of Vif and/or A3 plasmids. For HIV-luciferase transfections, 1 µg of HIV-Luc plasmid was used instead of both FIV plasmids. At 48 h posttransfection, cells and supernatants were collected.

A3 and Vif plasmids. All A3s are expressed as carboxy-terminal hemagglutinin (HA)-tagged proteins, except human (*Homo sapiens*) APOBEC3DE (HsaA3DE), a gift from Y.-H. Zheng, that carries a carboxy-terminal V5-tag (9) and human A3H splice variants (SV), gifts from Viviana Simon, that carry amino-terminal Flag tags (14). The human A3G expression construct was provided by N. R. Landau (25). FcaA3Z2s and -Z3, big cat (*Panthera tigris corbetti* [Pti], *Lynx lynx* [Lly], *Panthera leo bleyenberghi* [Ple], *Puma concolor* [Pco], *Panthera pardus japonensis* [Ppa])-A3Z2s and -Z3s, and *Equus caballus* (Eca)-, *Mus musculus* (Mmus)-, and *Sus scrofa* (Ssc)-A3 expression plasmids and human A3B, -C, and -F were previously described (22, 30, 31, 58). FcaA3Z2b-Z3 (SV-B), FcaA3Z2c-Z3 and big cat A3Z2-Z3 cDNAs were identified by reverse transcriptase PCR (RT-PCR) with the forward (fw) primer fAPO-30 (5-TGCATC GGTACCACCAAGGCTGGAGAGAGGAATGG-3) and the reverse (rv) primer fAPO-28 (5-AGCTCGAGTCAAGCGTAATCTGGAACATCGTATG GATATTCAAGTTTCAAATTTCTGAAG-3) using cDNA of total RNA from feline PBMC and *Pfu* polymerase (Fermentas, St. Leon-Rot, Germany) as desc ribed previously (31). Each 30 cycles were run at 94°C for 30 s, 58°C for 1 min, and 72°C for 2 min, and PCR products were cloned into the KpnI and XhoI sites of pcDNA3.1(+) and sequence verified. A3Z2c-Z3 variants including polymorphic sequences of exon 4 of four different *Felis catus* breeding lines were generated by exchanging the following amino acids through overlapping ext ension PCR: for Birman (BIR), G179A, F182S, and K186E; for Japanese Bobtail (BOB), K186E; for British Shorthair (SHO), R172Q, G179D, F182L, and K186E; for Turkish Van (VAN), K157E, H158Y, D165Y, H166N, and K186E (see Fig. 1B, top panel). The resulting full-length constructs were cloned into pcDNA3.1(+) using the KpnI and XhoI restriction sites. Human and feline A3Z2-Z3 chimera were made by exchanging the N-terminal or C-terminal or both zinc (Z)-coordinating domains of the FcaA3Z2c-Z3 template through overl apping extension PCR. The resulting cloned PCR products were sequence verified. The 5' and 3' fragments were amplified separately by using primer pairs fApo3F-18 x fe3C/hu3H.rv (5'-GTTAACAGAGCCATTGTGGGTCTGGGCA A-3') and fe3C/hu3H.fw (5'-TTGCCCAGACCCACAATGGCTCTGTTAAC-3') x huA3H-HA.rv (5'-TTCAGCTCGAGTCAAGCGTAATCTGGAACATC GTATGGATAGGACTGCTTTATCCTGTCAAG-3') for FcaA3Z2c-HsaA3Z3 (FcaA3Z2c-HsaA3H); CEM15-CEM13 (5'-TAAGCGGAATTCTATCTAAGAG GCTGAACATG-3') x hu3C/fe3H.rv (5'-CTTTGTTGGCCGGGATGGAG ACTCTCCCGT-3') and hu3C/fe3H.fw (5'-ACGGGAGAGTCTCCATCCCGG CCAACAAAG-3') x fAPO-28 for HsaA3Z2b-FcaA3Z3 (HsaA3C-FcaA3Z3) and the plasmids human A3C or A3H (HapII RDD-SV-183 [FJ376614] [14]) and FcaA3Z2c-Z3 as a template. To test the relevance of the interdomain linker of FcaA3Z2c-Z3, both feline domains were replaced by human sequences: HsaA3Z2b-HsaA3Z3 (HsaA3C-HsaA3H) was constructed by overlapping PCR using the primer pairs CEM15-CEM13 x fe3C/hu3H.rv and fe3C/hu3H.fw x huA3H-HA.rv and the plasmids human A3H (HapII RDD-SV-183) and HsaA3Z2b-FcaA3Z3 as a template. The 5 and 3 fragments were then mixed and amplified with the two external primers. The resulting full-length mutants were cloned into pcDNA3.1(+) using KpnI and XhoI restriction sites. V5-tagged $pVif_{HIV\text{-}1}$ of $HIV\text{-}1_{NL4\text{-}3}$ was generated by amplifying the *vif* gene from pNL-LucR$^-$E$^-$ (7) using fw primer HIV1_5_HH (5'-TGCAGGTACCATGGAAA ACAGATGGCAGGTGATGAT-3') and rv primer HIV1_3'_HH (5'-AATGG CGGCCGCTCACGTAGAATCCAGTCCCAAGAGCGGGTTTGGGATAG GCTTGCCGTGTCCATTCATTGTATGGCTC-3'). The amplicon was cloned into pcDNA3.1(+) carrying a *Woodchuck hepatitis virus* posttranscriptional regulatory element (WPRE) (11) via KpnI and NotI restriction sites. $pVif_{SIVagm}$ was provided by N. R. Landau and the V5 tag containing Vif of FIV ($pVif_{FIV}$) was described previously (31). $pVIF_{FIV}$ expresses the codon-optimized *vif* of FIV-34TF10, and it was generated by cloning the codon-optimized *vif* gene containing a V5 tag sequence at the 3 end, into pcDNA3.1(+) using the KpnI and NotI restriction sites; a 3'-WPRE element was included in the NotI and ApaI sites. Vif of FIV-34TF10 and Vif of FIV-PPR (expressed in the packaging construct pCPRΔEnv) share 90.4% identical amino acids. All sequences of plasmid DNA were obtained by dye terminator sequencing on an ABI 3730xl (Applied Biosystems, Darmstadt, Germany).

Viruses and infections. FIV single-cycle luciferase vectors (FIV-Luc) were produced by cotransfecting 293T cells with the following: the replication-deficient packaging construct pFP93 (derived from clone FIV-34TF10 [GenBank accession number M25381], a gift from Eric M. Poeschla [23]), which expresses *gag*, *pol*, and *rev* but does not express *env* or *vif* (referred as FIV*vif* in Results); the replication-deficient packaging construct pCPREnv (derived from clone FIV-PPR [GenBank accession number M36968], a gift from Garry P. Nolan [8]), which expresses all FIV genes, including Vif, but not *env* (referred as wt FIV in Results); the FIV luciferase vector pLiNSin (31); a VSV-G expression plasmid pMD.G; an A3 expression plasmid; or empty vector pcDNA3.1(+). Vector pLiNSin was derived from pGiNSin, a self-inactivating (Sin) vector variant of pGiNWF (23), which is a minimal bicistronic FIV transfer vector plasmid coding for enhanced green fluorescent protein (EGFP) and neomycin phosphotransferase containing the posttranscriptional regulatory element WPRE and the FIV central DNA flap. In addition, the luciferase reporter vectors FIV*vif* plus Vif were produced by cotransfecting 293T cells with pFP93, pLiNSin, a Vif expression plasmid (pMD.G), and an A3 expression plasmid or empty vector pcDNA3.1(+). Gag-Pol of strains FIV-34TF10 and FIV-PPR show ~96% identical amino acids. HIV-Luc *vif* was produced by cotransfecting 293T cells with pNL-LucR$^-$E$^-$*vif* (25) and pMD.G, together with defined A3 expression plasmidsor pcDNA3.1(+) and a Vif expression plasmid or empty vector pcDNA3.1(+). The RT activity of viruses was quantified by using the Cavidi HS Lenti RT kit (Cavidi Tech, Uppsala, Sweden). For reporter virus infections, HOS cells were seeded at 2.0 x 10^3 cells/well 1 day before transduction in 96-well plates and then infected with reporter virus stocks normalized for RT activity. Firefly luciferase activity was measured 3 days posttransduction with the Steadylite HTS reporter gene assay system (Perkin-Elmer, Cologne, Germany) according to the manufacturer's instructions on a MicroLumat Plus luminometer (Berthold Detection Systems, Pforzheim, Germany). Transductions were performed in triplicates; the means and standard deviations of each triplicate are shown. Replication-competent NL-BaL (NL4.3 with the *env* BaL) (26) virus stocks were prepared by harvesting the supernatant of transfected 293T cells. The cloning used to generate pNL-BaL.*vif*FIV and pNL-LucR$^-$E$^-$*vif*FIV results in an in-frame insertion of FIV *vif* in the HIV-1 *vif* coding region. The constructs were designed to express Vif_{FIV} by internal initiation. Three fragments were amplified separately using $pVIF_{FIV}$ and pNL-LucR$^-$E$^-$ as template DNA and the primer pairs Vpu_mut_5'out_HH (5'-GAACCGGTACATGGAGTGTATT AT-3') and HIV1_vif_FIV1.rv (5'-CTCTTCGCTCATGGCCTATGTATGCAG ACC-3'); HIV1_vif_FIV2.fw (5'-GGTCTGCATACATAGGCCATGAGCGAA GAG-3') and HIV1_vif_FIV2a.rv (5'-CTCCATTCTATGGTCAGGTGCTGTC C-3'); and HIV1_vif_FIV3.fw (5'-GGACAGCACCTGACCATAGAATGGA G-3') and HIV1_vif_FIV3.rv (5'-TGCAGAATTCTTATTATGGCTTCCA-3'). The forward primer HIV1_vif_FIV2.fw was used to insert a stop codon upstream of the FIV *Vif-V5* start codon. The fragments were fused through overlapping extension PCR, and the final fragment was amplified by using the forward Vpu_mut_5'out_HH and reverse HIV1_vif_FIV3.rv primers. The amplicon was cloned into restriction sites AgeI and EcoRI of the HIV-1 plasmids. The sequence identity of the final HIV-derived plasmids was confirmed: vif_{FIV} was inserted without disturbing the reading frames of the overlapping *pol* and *vpr*

genes. The final *vif* region encodes for the first 76 amino acids of Vif$_{HIV}$, followed by the *vif-V5* of FIV (see Fig. 5A for details). 3 of the stop codon of FIV *vif-V5*, the natural reading frame of the terminal 107 amino acids of *vif*$_{HIV}$ were maintained but are assumed to be nonfunctional. The kinetics of viral spreading replication was determined with feline CrFK.CD4.CCR5 cells, human HOS.CD4.CCR5 cells, and human PBMC by infection with 50-pg equivalents of RT from NL-BaL and NL-BaL.*vif*FIV. Spreading virus replication was quantified over 15 days using the Zeptometrix Retro-Tek HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) (Zeptometrix, New York, NY) according to the instructions of the manufacturer. For all transfections, transductions and infections, representative data are shown, and all experiments were repeated independently at least three times.

Immunoblot analysis. Transfected cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], protease inhibitor cocktail set III [Calbiochem, Darmstadt, Germany]). Virions (filtered, 0.45-μm pore size) culture supernatant) were pelleted by centrifugation through a 20% sucrose cushion at 35,000 rpm in a SW40 Ti rotor for 1.5 h and resuspended in RIPA buffer. The protein concentration of the lysates was quantified by using Bradford reagent (Applichem, Darmstadt, Germany). Then, 20 μg of each sample was separated by SDS-PAGE and transferred to polyvinylidene difluoride filters. Filters were probed with mouse anti-hemagglutinin (anti-HA) antibody (1:10,000 dilution, MMS-101P; Covance, Münster, Germany), mouse anti-Flag M2 antibody (1:1,000, clone M2; Sigma-Aldrich, Taufkirchen, Germany), mouse anti-V5 antibody (1:5,000 dilution, MCA1360; ABDserotec, Düsseldorf, Germany), mouse anti-α-tubulin antibody (1:4,000 dilution, clone B5-1-2; Sigma-Aldrich), mouse anti-capsid p24 hybridoma supernatant (1:50 dilution, α-p24 183-H12-5C; provided by Egbert Flory), or mouse anti-VSV-G antibody (1: 10,000 dilution; clone P5D4; Sigma-Aldrich), followed by horseradish peroxidase-conjugated rabbit anti-mouse antibody (α-mouse-IgG-HRP; GE Healthcare, Munich, Germany), and developed with ECL chemiluminescence reagents (GE Healthcare).

Statistical analysis. The data were analyzed by using a Dunnett *t* test using GraphPad InStat 3 software (GraphPad Software, San Diego, CA). This is a multiple-comparison method that uses a pooled standard deviation and allows a comparison of multiple values to a single control. *P* values of <0.05 were considered significant.

Data deposition. The novel sequences reported here have been deposited in the GenBank database (accession number): FcaA3Z2b-Z3 SV-B (HM100128), FcaA3Z2c-Z3 (GU097660), PtiA3Z2-Z3 (GU097663), PleA3Z2-Z3 (GU097662), LlyA3Z2-Z3 GU097661), and PcoA3Z2-Z3 (GU097659).

RESULTS

Complex splicing and readthrough transcription of feline A3s. Recently, we described five different *Felis catus* A3 cDNAs (FcaA3Z2a, -Z2b, -Z2c, -Z3, and the readthrough splice variant [SV]-Z2b-Z3 [now designated FcaA3Z2b-Z3 SV-A]) representing the genes of the sequenced Abyssinian cat genome (22, 31, 33). During the present study, we identified the alternative splice variant B (SV-B) of A3Z2b-Z3 with exons 1 to 4 of A3Z2b, whereas the previously described A3Z2b-Z3 splice variant A (SV-A) differs in exon 4 that is derived from the A3Z2c gene (Fig. 1A). Via RT-PCR we identified another two-domain A3 readthrough transcript in feline PBMC, A3Z2c-Z3. The A3Z2c-Z3 cDNA is composed of the fused open reading frames of A3Z2c and A3Z3 (Fig. 1A). All readthrough A3s differ moderately from each other in three or six amino acids. In these two-domain feline A3 proteins, the Z2 and Z3 domains are naturally separated by a linker region that is derived from the 156-nucleotide (nt) long unique 5' region of the A3Z3 Exon 2 (Fig. 1). In addition, we cloned expression plasmids of A3Z2c-Z3s containing the polymorphic sequences detected in exon 4 of A3Z2c of the four domestic cat breeds Japanese Bobtail (BOB), Birman (BIR), British Shorthair (SHO), and Turkish Van (VAN) (31). The exon 4-derived variability of the cat breeds with respect to the reference Abyssinian sequence affected up to five amino acids (Fig. 1B, top panel; for details, see Materials and Methods).

FIV Vif broadly counteracts *Felidae* A3s. To test these A3s against FIV, we analyzed the infectivity of wt, Δ*vif* FIV, and Δ*vif* FIV plus Vif$_{FIV}$ produced in the presence of the described feline A3s and human A3G using single-round luciferase reporter vectors. In the wt FIV vector system (based on FIV-PPR), the Vif protein is expressed in its natural genomic context, whereas the Δ*vif* FIV vector system (based on FIV-34TF10) lacks the *vif* gene. To complement Vif in *trans*, plasmid pVif$_{FIV}$ expressing a codon-optimized Vif protein of FIV-34TF10 with a C-terminal V5 tag was used. The Vifs of FIV-34TF10 and of FIV-PPR share 90.4% identical amino acids and Gag-Pol proteins of both FIV strains are ~96% identical.

293T cells were cotransfected either with wt FIV or Δ*vif* FIV or Δ*vif* FIV plus the Vif$_{FIV}$ expression plasmid and one of the A3 expression plasmids. At 2 days posttransfection, virus-containing cell supernatants were collected, normalized for RT activity, and used to transduce HOS cells. The intracellular luciferase activity in transduced HOS cells was analyzed 3 days postinfection (dpi), a time when provirus formation had finished. Similar to recent findings (31), expression of the three A3Z2 isoforms did not inhibit wt or Δ*vif* FIV (compare the first bar with the second and third bars in sets II to IV, Fig. 2A; variations are not statistically significant). A3Z3 reduced the infectivity of Vif-deficient FIV ~5-fold (statistically significant, $P < 0.01$), but not if Vif was expressed (compare the first bar with the second and third bars in set V, Fig. 2A). Both the Vif of the wt FIV packaging construct and of the Vif$_{FIV}$ expression plasmid induced a depletion of A3Z2s and of A3Z3 proteins in the producer cells, indicating that the V5 tag in Vif did not compromise the Vif function (compare the first lane with the second and third lanes in sets II to IV of cell lysate, Fig. 2B). Interestingly, not only A3Z3 also A3Z2 proteins were easily detectable in particles of Δ*vif* FIV (see first lane of set II to V, virion lysates, Fig. 2B). In analyzing the double-domain A3s, we found that both A3Z2-Z3s were active against Δ*vif* FIV (6- to 12-fold inhibition; $P < 0.01$) but were completely or mostly inactive when Vif was coexpressed (compare the first bar with the second and third bars in sets VI and VII, Fig. 2A). A3Z2b-Z3 splice variants A and B showed indistinguishable expression levels and activities (data not shown); therefore, all figures show only experiments performed with the A3Z2b-Z3 (SV-A).

The high genetic variability in exon 4 of A3Z2c in some cat breeds (31) made us wonder whether these amino acids are the result of a positive selection in response to FIV. We found that the A3Z2c-Z3 constructs called BIR, BOB, SHO, and VAN (see above) were at least as active as the parental A3Z2c-Z3 (Abyssinian cat) under the experimental conditions. The A3 constructs containing the exon 4 sequences from BIR, SHO, and VAN significantly inhibited up to 15-fold, and the most active variant with sequences of BOB reduced the infectivity of Δ*vif* FIV by 38-fold (first bar in sets IX, XI, and XII, Fig. 2A). Coexpression of Vif$_{FIV}$ fully counteracted the A3Z2c-Z3s from the Abyssinian cat and constructs with sequences of BIR and SHO (second and third bars in sets IX and XI, Fig. 2A) and mostly rescued FIV also from A3Z2-Z3s with the exon 4 variability of BOB, with a difference of no statistical significance

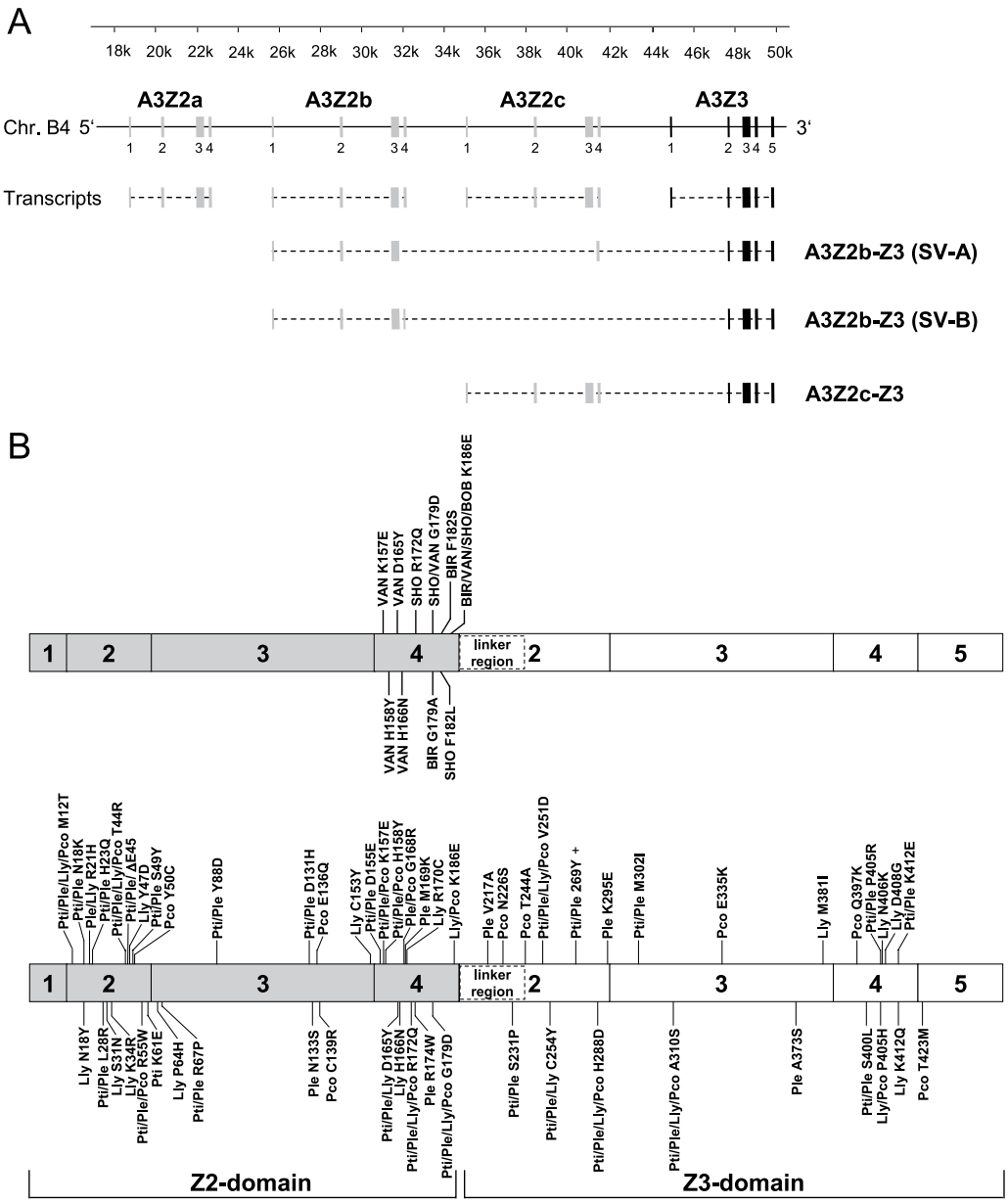
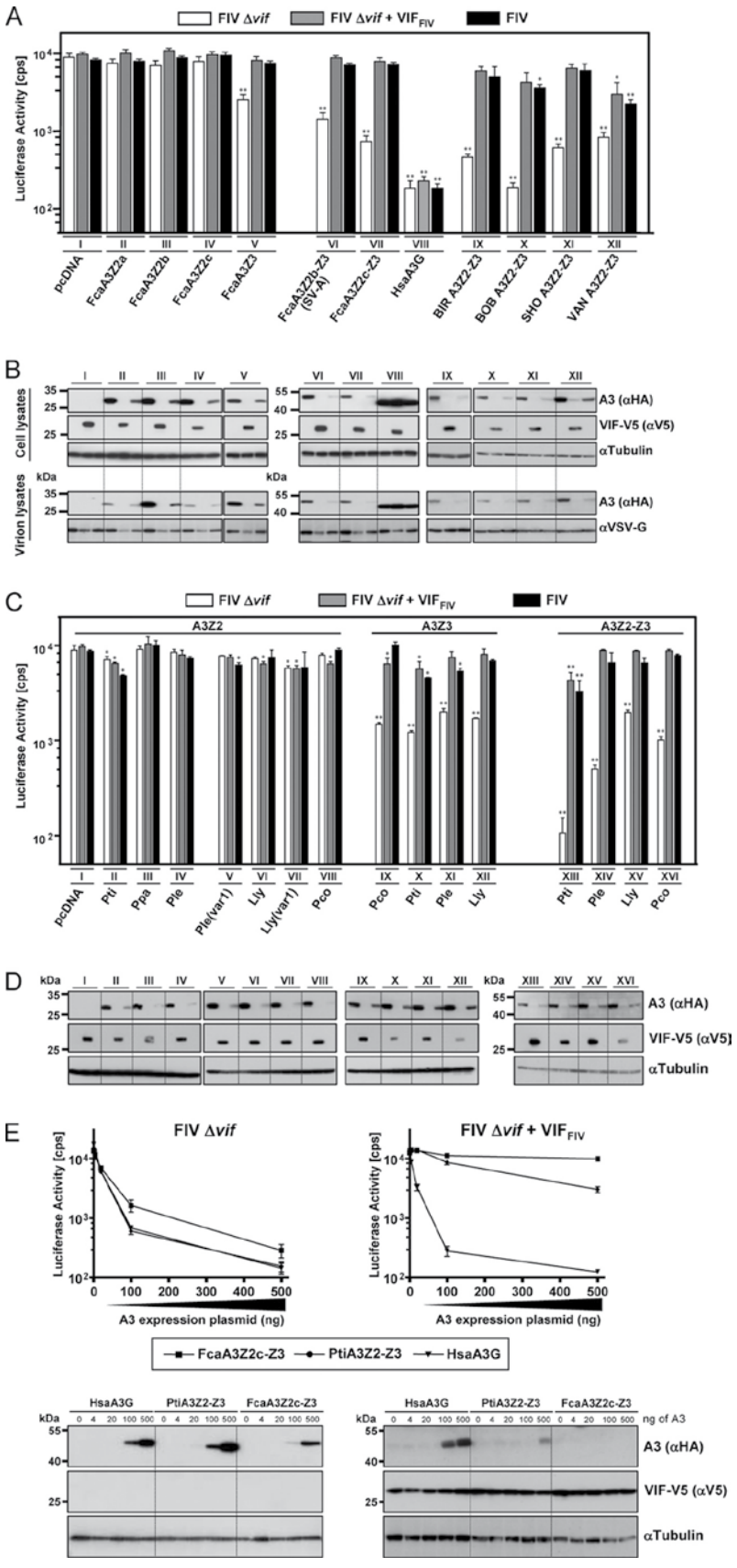


FIG. 1. Representation of APOBEC3 (A3) coding regions in the genome of *Felis catus*. (A) Transcripts with translated exons (Z2, gray rectangles; Z3, black rectangles) and spliced-out introns (dashed lines) are indicated. Three readthrough transcripts were found: the already described two-domain FcaA3Z2c-Z3 (31) and the two splice variants (SV) A3Z2b-Z3 (SV-A) and A3Z2b-Z3 (SV-B) that include the exon 4-derived sequence of either A3Z2b or -Z2c in an additionally spliced version (for details, see reference 31). (B) Schematic representation of the exon structure of the A3Z2-Z3 readthrough transcripts. The locations of amino acid replacements in FcaA3Z2c-Z3 are indicated with respect to exon 4 variability of major cat breeds (upper figure) and A3Z2-Z3 genes of big cats (lower figure). A plus sign indicates the insertion of an amino acid. Numbers in shaded boxes indicate exons from Z2c, and those in open boxes indicate exons from Z3. The region of Z2-Z3 proteins designated "linker region" protein is encoded by exon 2 of A3Z3, a sequence that is untranslated in single-domain A3Z3. BIR, Birman; BOB, Japanese Bobtail; SHO, British Shorthair; VAN, Turkish Van; Pti, *Panthera tigris corbetti*; Ppa, *Panthera pardus japonensis*; Ple, *Panthera leo bleyenberghi*; Lly, *Lynx lynx*; Pco, *Puma concolor*.

for *vif* FIV plus Vif$_{FIV}$ and significance for wt FIV ($P < 0.05$) (second and third bars, set X, Fig. 2A). Vif could not completely restore the infectivity of Δ*vif* FIV+Vif$_{FIV}$ or wt FIV made in the presence of VAN (statistically significant, $P < 0.05$ or $P < 0.01$, respectively), and it remained reduced by ~5-fold (second and third bars, set XII, Fig. 2A). Immunoblots of the virus producer cells demonstrated equal expression of the A3 proteins and a Vif-dependent depletion (compare the first lane


A
FIV Δvif
FIV Δvif + VIF FIV
FIV
Luciferase Activity [cps]
pcDNA
FcaA3Z2a
FcaA3Z2b
FcaA3Z2c
FcaA3Z3
FcaA3Z2b-Z3 (SV-A)
FcaA3Z2c-Z3
HsaA3G
BIR A3Z2-Z3
BOB A3Z2-Z3
SHO A3Z2-Z3
VAN A3Z2-Z3
B
Cell lysates
Virion lysates
kDa
A3 (αHA)
VIF-V5 (αV5)
αTubulin
αVSV-G
C
A3Z2
A3Z3
A3Z2-Z3
pcDNA
Pti
Ppa
Ple
Ple(var1)
Lly
Lly(var1)
Pco
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E
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HsaA3G
ng of A3

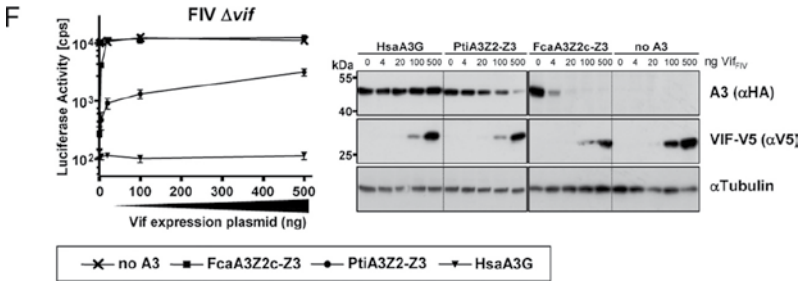

FIG. 2. FIV Vif of domestic cats overcomes the antiviral activity of *Felidae* A3s. VSV-G pseudotyped wt FIV (expressing Vif), Δ*vif*, and Δ*vif* plus VifFIV luciferase reporter vectors were produced in 293T cells cotransfected with HA-tagged A3 expression plasmids of the domestic cat and chimeric A3s with major amino acid exchanges of exon 4 of A3Z2c of four cat breeding lines (A and B) and big cats (C and D). Roman numbers indicate a set of transfections with the same A3 plasmid. Plasmid pVifFIV expresses a V5-tagged Vif protein. pcDNA3.1(+) (pcDNA) was included as a control. Reporter vector infectivity was determined by quantification of luciferase activity in HOS cells transduced with vector particles normalized for RT. Asterisks represent statistically significant differences (∗, $P < 0.05$; ∗∗, $P < 0.01$ [Dunnett *t* test]) relative to the pcDNA control. A3 and Vif (Vif-V5) expression in the transfected 293T producer cells were detected by immunoblotting with anti-HA antibody for A3 or anti-V5 for Vif (B and D). Cell lysates were also analyzed for equal amounts of total proteins by using anti-tubulin antibody. For panel B, *Felis catus* A3s proteins were also analyzed for encapsidation into released FIV particles. Encapsidated A3 proteins were detected on immunoblots probed with anti-HA antibody. Vector particle lysates were analyzed for equal amounts of viral proteins by using anti-VSV-G antibody. (E) Increasing amounts of A3 (0, 4, 20, 100, and 500 ng of plasmid) were tested against Δ*vif* FIV and Δ*vif* FIV plus Vif (500 ng of pVifFIV plasmid) and alternatively Δ*vif* FIV in the presence of 500 ng of A3 plasmid with increasing amounts of VifFIV (0, 4, 20, 100, and 500 ng of plasmid) was analyzed (F). Corresponding immunoblots of lysates of the vector producer cells are shown. A3 and Vif (Vif-V5) expression in transfected 293T producer cells were detected by immunoblotting with anti-HA antibody for A3 or anti-V5 for Vif. Cell lysates were also analyzed for equal amounts of total proteins by using anti-tubulin antibody. α, anti; cps, counts per second; BIR, Birman; BOB, Japanese Bobtail; SHO, British Shorthair; VAN, Turkish Van; Fca, *Felis catus*; Pti, *Panthera tigris corbetti*; Ppa, *Panthera pardus japonensis*; Ple, *Panthera leo bleyenberghi*; Lly, *Lynx lynx*; Pco, *Puma concolor*; Hsa, *Homo sapiens*; (var1), A3Z2 variant transcript detected in the indicated species.

with the second and third lanes in sets II to VII and IX to XII, Fig. 2B). As expected, the antiviral activity of HsaA3G was not counteracted by Vif$_{FIV}$. Taken together, these findings suggest that the genetic variability in exon 4 of A3Z2c-Z3 has a minor effect on the infectivity of domestic cat FIV. However, amino acids derived from exon 4 of Turkish Van do have some impact on the counteraction of Vif$_{FIV}$. Because our studies, described below, implicate that Vif$_{FIV}$ interacts with both Z domains of A3Z2c-Z3, the mechanism of the moderate Vif resistance of VAN cannot be explained and requires further investigation.

Our finding that Vif of FIV potently inhibited mostly all tested A3s of the domestic cat prompted us to evaluate the ability of Vif$_{FIV}$ to counteract A3 proteins of felid species different from the domestic cat lineage. For this purpose, A3Z2, A3Z3, and A3Z2-Z3 expression plasmids of *Panthera tigris corbetti*, *Lynx lynx*, *Panthera leo bleyenberghi*, *Puma concolor*, and A3Z2 of *Panthera pardus japonensis*, representing three lineages within *Felidae*, were analyzed for their anti-FIV activity. Because only the *F. catus* chromosomal A3 locus is characterized (31), the felid A3 cDNAs of big cats cannot be related to a specific Z2 gene or to a specific Z2-Z3 readthrough transcript. Comparing the protein sequence of FcaA3Z2c-Z3 to those of big cats, many amino acids changes become obvious, especially in exons 2 and 4 of A3Z2 and exon 4 of A3Z3 (Fig. 1B, bottom panel). Testing the big cat A3s in the FIV luciferase vector system, we surprisingly detected no strong inhibitory activity against the wt domestic cat FIV (see the second and third bars in sets II to XVI, Fig. 2C). The felids A3Z2s did not much affect the infectivity of wt or *vif* FIV (sets II to VIII, Fig. 2C) similar to the inert antiviral activities of the domestic cat counterparts (sets II to IV, Fig. 2A). However, we found for some felid A3Z2s a mild, sometimes significant, inhibition of ~1.5- to 2-fold of FIV (see sets II to VIII, Fig. 2C). Vif-deficient FIV was significantly ($P < 0.01$) inhibited by A3Z3s and A3Z2-Z3s, but in all cases the antiviral activity was efficiently suppressed by Vif (compare the first bar to the second and third bars, set IX to XVI, Fig. 2C). Compared to the infectivity data (Fig. 2C), Vif induced a robust depletion of all big cat A3s in the corresponding producer cells (compare the first lane to the second and third lanes in sets II to XVI, Fig. 2D). The tiger A3s were slightly, but significantly, more active than those of the other big cat A3s (see sets II, X, and XIII, Fig. 2C), such as PtiA3Z2-Z3, inhibited in this experiment the infectivity of *vif* FIV by 85-fold and inhibited FIV ca. 3- to 5-fold despite the coexpression of Vif ($P < 0.01$, compare the first bar to the second and third bars, set XIII, Fig. 2C).

To demonstrate that the moderate inhibition of FIV by tiger A3Z2-Z3 is relevant, a titration of cat Vif and tiger A3 plasmids in the context of *vif* FIV was performed (Fig. 2E and F). In contrast to the standard assays, where each 500 ng of pVif$_{FIV}$ and A3 expression plasmids were transfected, 4, 20, 100, or 500 ng each of FcaA3Z2c-Z3, PtiA3Z2-Z3, and Vif$_{FIV}$ was used. Human A3G was included as a control for a Vif$_{FIV}$-

insensitive A3. The titration curves of human A3G and tiger A3 in the absence of Vif were very similar to each other and showed that in the range from 100 to 500 ng of plasmid tiger A3Z2-Z3 and A3G were ca. 2- to 3-fold more inhibitory than FcaA3Z2c-Z3 (Fig. 2E, left panel). We redid these experiment in the presence of 500 ng of Vif expression plasmid (Fig. 2E, right panel) and saw that only the highest amount of the tiger A3Z2-Z3 plasmid (500 ng) inhibited FIV by ~5-fold.

To further study whether a reduced Vif expression would render FIV replication more vulnerable to A3 restriction, a fixed amount of 500 ng of A3 expression plasmid and varied concentrations of the Vif plasmid were used (Fig. 2F). We found that FcaA3Z2c-Z3 was very sensitive to Vif and that 20 ng of pVif$_{FIV}$ could fully restore FIV infectivity. In stark contrast, the heterologous tiger A3Z2-Z3 inhibited FIV ~10-fold in the presence of low amounts of Vif (20 and 100 ng of Vif plasmid). Only with the highest amount of pVif$_{FIV}$ (500 ng) was the antiviral activity of tiger A3Z2-Z3 reduced to an ~4-fold inhibition (Fig. 2F). The corresponding immunoblots of the vector producer cells (Fig. 2F) showed that the degree of Vif-induced depletion of the tiger and cat A3Z2-Z3s correlated with the differences of these A3 to inhibit FIV. Whereas 20 ng of pVif$_{FIV}$ was sufficient to deplete all detectable levels of FcaA3Z2c-Z3, small amounts of tiger A3Z2-Z3 were detectable even in the presence of 25-fold more Vif plasmid (Fig. 2F). Together, these results indicate that, depending on the expression levels of A3 and of Vif, even a moderate Vif-insensitive A3 (such as the tiger A3Z2-Z3) weakly inhibits FIV replication. However, considering the results of the experiments with the wt FIV vector system that expresses in *cis* natural amounts of Vif (set XIII in Fig. 2C), it is likely that FIV under *in vivo* conditions could counteract most of the inhibitory effect of tiger A3Z2-Z3. Thus, we conclude that, in contrast to the reported species-specific interaction of Vif$_{HIV-1}$ to human but not to nonhuman primate A3Gs (25, 52), Vif$_{FIV}$ showed barely any species specificity for felid A3s (Fig. 2C). More studies are required to determine whether Vif expression levels vary during natural infections, e.g., due to the presence or absence of cellular host factors for splicing or posttranslational protein modifications, and whether these variations are relevant to preventing cross-species transmissions among felids.

Nonfelid A3s are resistant to Vif of FIV. To further characterize the specificity of Vif$_{FIV}$, we tested A3s from human and other nonfelid species. These experiments were also done to identify the nonfelid A3s that likely restrict cross-species transmission of FIV *in vivo*. We generated FIV particles as described above together with one of the human, equine, porcine, or murine A3 expression plasmids. Again, HOS cells were transduced with the FIV vector normalized for RT, and at 3 dpi the intracellular luciferase activities were quantified. As shown in Fig. 3, human A3F, A3G, and haplotype (hap) and splice variants (SV) of A3H, equine A3Z3, porcine A3Z2-Z3, and murine A3Z2-Z3 proteins were found to exert a significant inhibitory effect on the infectivities of wt and *vif*-deficient FIV. In contrast to the activity of Vif against the antiviral effect of A3s from *Felidae* (Fig. 2A and C), all nonfelid A3s showed resistance to Vif$_{FIV}$ (Fig. 3). Although human A3A, A3B, A3C, and A3DE did not restrict FIV, we found that human A3H alleles encoding a single nucleotide polymorphism cluster (G105R, K121D, E178D, hapII-RDD) restricted FIV more efficiently than "wild-type" A3H (hapI-GKE), which is very similar to the described restriction pattern against HIV-1 (14). The activity of A3H against FIV was independent of the tested splice isoforms (SV-182, SV-183, and SV-200 [14]). Under these experimental conditions, the human A3G reduced the infectivity of FIV by 130-fold, human A3F and A3H (hapI-GKE)by ~10-fold, and pig and mouse A3Z2-Z3 by 25-fold, and the hapII-RDD variant of the human A3H reduced FIV infectivity by 60-fold. The equine A3Z1b, A3Z2-Z2, and A3Z2e proteins moderately inhibited FIV only 2- to 3-fold. Together, the results demonstrate that humans, horses, mice, and pigs have at least one A3 gene that likely forms part of the barrier against a cross-species transmission of FIV.

The double-domain FcaA3Z2c-Z3 protein contains two Vif interaction sites. In order to induce proteasomal degradation, Vif$_{HIV-1}$ binds either the C-terminal Z2f domain of human A3F (HsaA3Z2e-Z2f) or the N-terminal Z2g domain of human A3G (HsaA3Z2g-Z1c) (17, 42, 56, 57). Protection of FIV replication by Vif$_{FIV}$ is always associated with depletion of the antiviral feline A3 proteins, and Vif$_{FIV}$ can even induce depletion of the feline A3 proteins that do not restrict FIV (Fig. 2B, compare A3 detection in cell lysates in the first, second, and third lanes in sets II, III, and IV). These findings suggest that Vif$_{FIV}$ can interact with feline Z3 and Z2 domains.

To test this hypothesis, Vif interaction with FcaA3Z2c-Z3 (Abyssinian breed) was assayed using feline and human A3Z2-Z3 chimeras (Fig. 4A). For chimera FcaA3Z2c-HsaA3H (FcaA3Z2c-HsaA3Z3), the feline A3Z3 was substituted by HsaA3H (HsaA3Z3, hapII-RDD SV-183). Conversely, in HsaA3C-FcaA3Z3 (HsaA3Z2b-FcaA3Z3), the N-terminal feline A3Z2 was exchanged by human A3C (HsaA3Z2b). To test the relevance of the interdomain linker of the parental feline protein, both feline domains were replaced by human sequences, termed HsaA3C-HsaA3H (HsaA3Z2b-HsaA3Z3) (Fig. 4A). These A3s, together with A3Gs of human and AGM origin, were tested by using the single-cycle Δ*vif* FIV- and Δ*vif* HIV-1-luciferase reporter viruses to determine their antiviral activities and their susceptibilities to FIV, HIV-1, and SIVagm Vif proteins. Viral vector stocks were produced in 293T cells, and the infectivities of normalized particles were measured on HOS cells by quantification of the intracellular luciferase activity.

All A3 chimera inhibited both viruses in the absence of Vif proteins (Fig. 4B and C, compare the first bars in sets I, II, III, and IV). Vif$_{FIV}$ counteracted FcaA3Z2c-HsaA3H and HsaA3C-FcaA3Z3, but not HsaA3C-HsaA3H, in both viral systems (see the second bars in sets II, III, and IV of Fig. 4B and C). The Vif of HIV-1 failed to inhibit feline-human chimera FcaA3Z2c-HsaA3H (compare the first and third bars in set II of Fig. 4B and C), the wt feline A3Z2c-Z3, and the agmA3G (see the third bars in sets V and VII, Fig. 4B and C). These results were expected because human A3H, feline A3s, and agmA3G are resistant to Vif of HIV-1 (14, 25, 33). The human-feline (HsaA3C-FcaA3Z3) and the human-human (HsaA3C-HsaA3H) chimeras were sensitive to Vif$_{HIV-1}$ in the HIV vector system (see the third bars in sets III and IV, Fig. 4C). However, in the FIV vector system Vif$_{HIV-1}$ insufficiently counteracted the human-feline A3 (HsaA3C-FcaA3Z3) and, like Vif$_{SIVagm}$, only weakly inhibited the human-human (HsaA3C-HsaA3H) A3 chimera (compare third bar in set III and IV of Fig. 4B and 4C). These unexpected results implicate

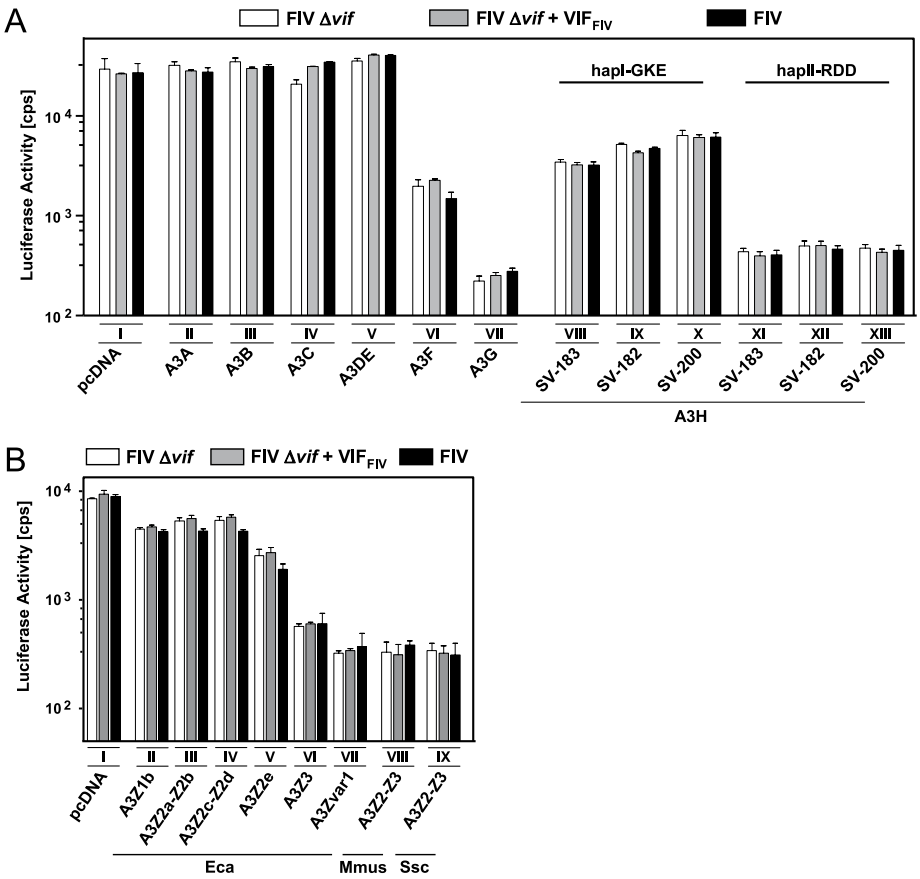

FIG. 3. Inhibition of FIV by nonfeline A3s. VSV-G pseudotyped FIV luciferase reporter vectors (wt FIV, expressing Vif; Δ*vif*; and Δ*vif* plus Vif_{FIV}) were produced in 293T cells in the presence or absence (pcDNA) of human A3s (A) or nonprimate equine, murine, and porcine A3s (B). The infectivity of the vector particles was determined by quantification of luciferase activity in HOS cells transduced with equal amounts of particles. pcDNA, pcDNA3.1(+); SV, splice variant; hapI, haplotype I; hapII, haplotype II; cps, counts per second; Eca, *Equus caballus*; Mmus, *Mus musculus*; Ssc, *Sus scrofa*. Roman numerals indicate a set of transfections with the same A3 plasmid.

that Vif of HIV-1 can interact with the A3C domain if it is part of an engineered double-domain A3 and that this interaction can be modulated by viral proteins of the packaging constructs. It is thus possible that conformational changes in the human-feline A3 (HsaA3C-FcaA3Z3) required for Vif_{HIV-1} interaction are induced or stabilized by binding of this A3 protein to HIV-1 Gag but not by binding to FIV Gag. The SIVagm Vif acted as an active inhibitor of agmA3G, human-feline A3 (HsaA3C-FcaA3Z3), feline-human A3 (FcaA3Z2c-HsaA3H) in both viral vector systems, but was less efficient against the human-human (HsaA3C-HsaA3H) chimera (compare the fourth bars in Fig. 3B and C). Although the results support that Vif_{FIV} can use both Z domains to inhibit feline single- and double-domain A3s, conformational problems of the human and feline chimeras might have influenced the sensitivity to Vifs of HIV-1 and SIVagm. Taken together, we conclude that both Z domains of FcaA3Z2c-Z3, but not the linker region, interact with Vif_{FIV}.

FIV Vif protects HIV-1 in feline cells. To corroborate the observation that Vif_{FIV} can protect Δ*vif* HIV-1 against feline A3s (see the second bar in set V, Fig. 4C), we sought to determine whether Vif_{FIV} expressed in *cis* in the proviral context would also rescue HIV-1 from feline A3s. Therefore, two HIV-1-derived constructs, termed NL-LucR$^-$E$^-$*vif*FIV (HIV-Luc *vif*FIV) and NL-BaL.*vif*FIV, were generated, in which the HIV-1 *vif* gene was replaced by the codon-optimized FIV-34TF10 *vif* gene expressed by internal initiation (for details of the construction, see Materials and Methods and Fig. 5A).

To quantify protection by Vif_{FIV} in *cis*, we compared the infectivities of the HIV-1 luciferase reporter viruses NL-LucR$^-$E$^-$Δ*vif* and NL-LucR$^-$E$^-$.*vif*FIV. The data in Fig. 5B show that in human cells the six feline A3 proteins inhibited Δ*vif* HIV-1-mediated gene transfer to different degrees, depending on the type of A3 protein, similar to previous results (33). FcaA3Z2a isoforms reduced the infectivity of Δ*vif* HIV-Luc by 3- to 4-fold; A3Z3 inhibited the infectivity by 6-fold, and the double-domain A3Z2-Z3 proteins inhibited the infectivity by 14- to 33-fold. Importantly, Vif_{FIV} expressed by the chimeric HIV-Luc fully protected HIV-1 against the antiviral activity of all feline A3s under these experimental conditions (see the second bars in sets II to VII, Fig. 5B). In these cases, the expression of Vif_{FIV} induced the depletion of feline A3s in

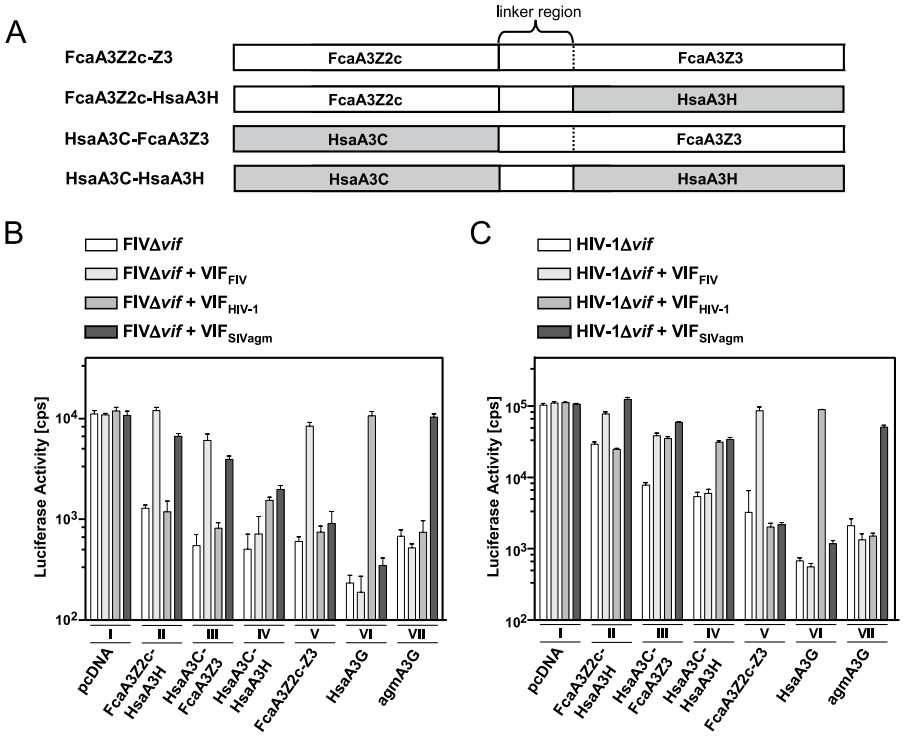

FIG. 4. Analyses of human/feline A3 chimeras reveal Vif interactions with both Z-domains of FcaA3Z2c-Z3. (A) Schematic representation of human/feline A3Z2-Z3 chimera. Open bars denote feline sequences; filled bars denote human sequences. In FcaZ2-Z3, the Z2 and Z3 domains are naturally separated by a linker region (demarcated by dotted lines) that is encoded by exon 2 of Z3, which is untranslated in FcaA3Z3 (see Fig. 1A). FIVΔ*vif* (B) and HIVΔ*vif* (C) luciferase reporter particles were produced in 293T cells in the presence or absence (pcDNA) of feline and chimeric human/feline A3Z2-Z3s expression plasmids and expression plasmids for Vif_{FIV}, Vif_{HIV-1}, or Vif_{SIVagm}. To control the specificity of primate Vifs, human and AGM A3G expression plasmids were included. The infectivities of the vector particles were determined by quantification of luciferase in HOS cells transduced with equal amounts of vector particles. pcDNA, pcDNA3.1(+); cps, counts per second. Roman numerals indicate a set of transfections with the same A3 plasmid.

the producer cells and likely thereby rescued vector infectivity (compare A3 detection in cell lysates in the first and second lanes of sets II to VII in Fig. 5C). A3-mediated restriction of marker gene expression was found to correlate with the presence of A3Z3 and A3Z2-Z3 proteins in released particles (see A3 detection in virion lysates in the first lanes of sets V, VI, and VII in Fig. 5C). The moderately active A3Z2s were only weakly detectable in lysates of the virions (see A3 detection in virion lysates, first lanes of sets II, III and IV, Fig. 5C). Human A3G was encapsidated in both particles, Δ*vif* HIV-Luc and HIV-Luc *vif*FIV (see A3 detection in virion lysates in set VIII in Fig. 5C). Also, both reporter viruses were equally inhibited by A3G (see both bars in set VIII of Fig. 5B), thereby strongly supporting that HIV-Luc *vif*FIV does not express any residual Vif_{HIV-1} activity.

Next, we evaluated the ability of Vif_{FIV} to support spreading replication of HIV-1 in feline cells. Wild-type HIV-1 (NL-BaL) and NL-BaL.*vif*FIV were used to infect feline CrFK.CD4.CCR5 and human HOS.CD4.CCR5 cells that are both genetically modified to express human CD4/CCR5. In addition, we infected PHA/IL-2-activated human PBMC with both viruses. From previous experiments, we knew that in contrast to human PBMC, HOS.CD4.CCR5 cells do support replication of Δ*vif* HIV-1 (data not shown). Feline CrFK or CrFK.CD4.CCR5 cells support replication of neither *vif* FIV nor wt HIV-1 (33, 38, 44). In the supernatants of infected cells, the amount of released CA.p24 was measured over a period of 15 days. The results shown in Fig. 5D demonstrated that wt HIV-1 replicated efficiently in HOS.CD4.CCR5 cells and in human PBMC but not in the CrFK.CD4.CCR5 cells (Fig. 5D). In contrast, the Vif_{FIV}-encoding virus (NL-BaL.*vif*FIV) was able to replicate to similar levels in the permissive human HOS.CD4.CCR5 and the nonpermissive feline CrFK.CD4.CCR5 cells (Fig. 5D). Human PBMC did not support efficient spreading of NL-BaL.*vif*FIV (Fig. 5D). The PBMC cultures showed highest permissivity toward wt HIV-1 but yielded in very low p24 levels in the supernatant after inoculation with NL-BaL.*vif*FIV (Fig. 5D), which may be derived from the initial first round of infection. These data confirm (see above) that the Vif_{FIV}-chimeric viruses carry a HIV *vif* gene that is genetically and functionally fully inactivated. Expression of Vif_{FIV} was confirmed in virion lysates of the infected HOS.CD4.CCR5 and CrFK.CD4.CCR5 cultures in which NL-BaL.*vif*FIV showed spreading replication (Fig. 5E). At 12 dpi, the signal for the Vif-V5 was weaker than the V5 signal of samples of day 5 in both cultures, which may be due to a partial loss of the nonessential V5 tag of the Vif_{FIV} protein. We conclude that, at least in some feline cell types, A3 proteins constitute the major restriction mechanism acting inhibitory against HIV-1.

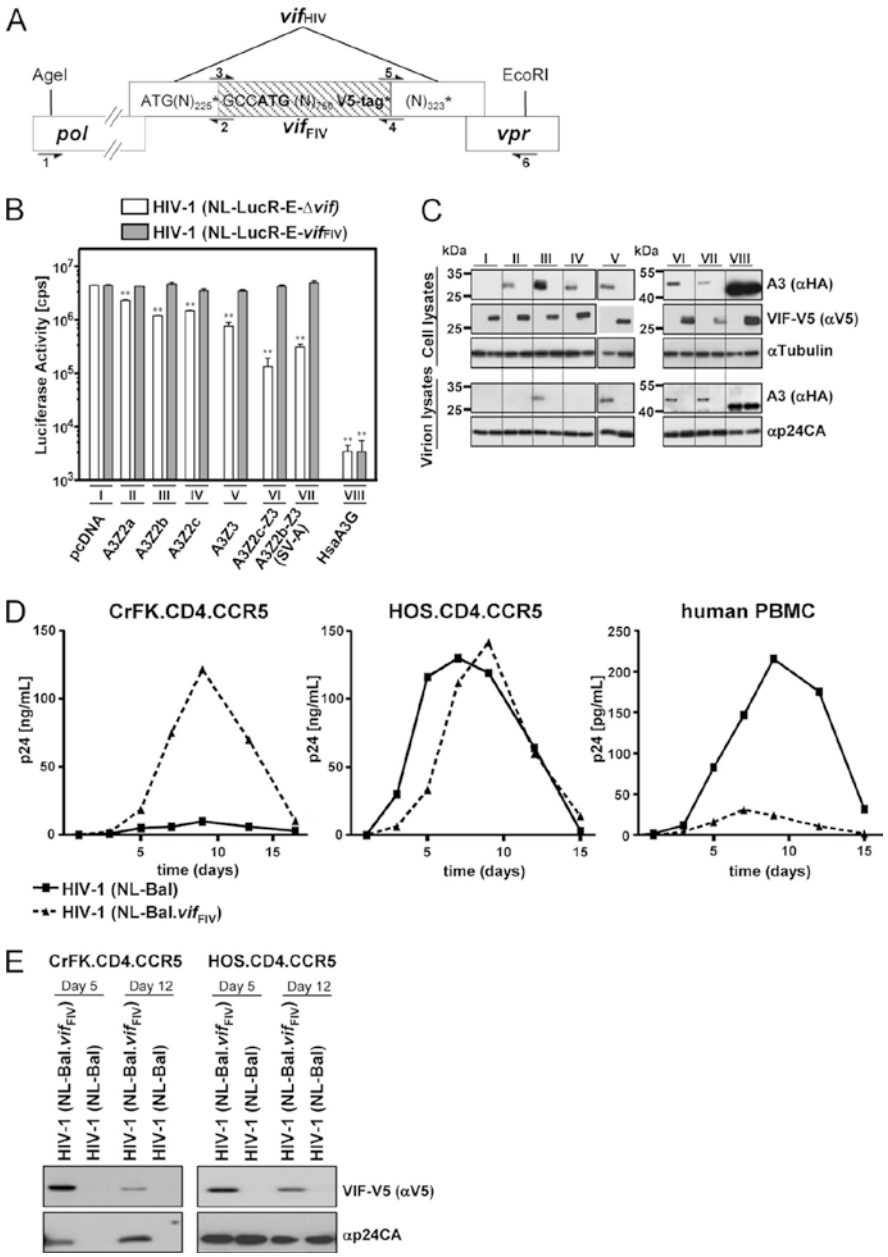

FIG. 5. HIV-1 is protected by VifFIV in feline cells. (A) Schematic representation of the insertion of the vif_{FIV} (shaded) in the *vif* gene of the HIV-derived constructs (NL-LucR$^-$E$^-$$vif_{FIV}$ and NL-Bal.vif_{FIV}) by fusion PCR. Overlapping *pol* (schematically depicted) and *vpr* genes are shown. Restriction enzyme recognition sites AgeI and EcoRI were used to clone the fusion PCR product. Position of PCR primers used are shown as arrows: primer 1, vpu_mut_5'out_HH; primer 2, HIV1_vif_FIV1.rv; primer 3, HIV1_vif_FIV2.fw; primer 4, HIV1_vif_FIV2a.rv; primer 5, HIV1_vif_FIV3.fw; and primer 6, HIV1_vif_FIV3.rv. "N" represents adenine (A), cytosine (C), guanine (G), or thymidine (T). $(N)_{number}$ represents the number of Ns. *, Stop codon. (B and C) The effect of Vif_{FIV} in *cis* for the infectivity of the chimeric HIV-1 constructs was tested by using single-cycle luciferase reporter viruses. VSV-G pseudotyped virus was produced by cotransfection of pNL-LucR$^-$E$^-$Δ*vif* or pNLLucR$^-$E$^-$*vif*FIV in the presence or absence (pcDNA) of the indicated expression plasmids for feline A3 and human A3G. Infectivity of the viruses was determined by quantification of luciferase in HOS cells infected with normalized amounts of viruses (B). Asterisks represent statistically significant differences (*, $P < 0.05$; **, $P < 0.01$ [Dunnett *t* test]) relative to the pcDNA control. Corresponding immunoblots of cell lysates and virion lysates were probed with anti-HA antibody for A3 expression (C). The expression of Vif_{FIV} was confirmed by probing the blot with an anti-V5 antibody. Cell lysates were also analyzed for equal amounts of total proteins by using an anti-tubulin antibody. Virus lysates were analyzed for equal amounts of viral proteins by using anti-p24CA (HIV-1 capsid) antibody. Roman numbers indicate a set of transfections with the same A3 plasmid. (D and E) Replication of wild-type HIV-1(NL-Bal) and HIV-1(NL-Bal.vif_{FIV}). Human HOS.CD4.CCR5 cells, feline CrFK.CD4.CCR5 cells, and PHA/IL-2 activated human PBMC were infected with NL-BaL and NL-BaL.vif_{FIV} at a multiplicity of infection of 0.05. (D) Culture supernatants were quantified every 2 or 3 days by p24CA ELISA. (E) Immunoblot analysis of virus lysates from infected CrFK.CD4.CCR5 and HOS.CD4.CCR5 cells on days 5 and 12 postinfection. Expression of Vif_{FIV} was confirmed by probing the filter with an anti-V5 antibody, the same blot was also stained with anti-p24CA (capsid) antibody to detect HIV-1. α, anti.

DISCUSSION

Examples of FIV cross-species transmissions have been reported and are currently studied to gain increased understanding regarding the permissiveness of selected and sometimes endangered feline species to diverse, molecularly characterized isolates of pathogenic FIV. These studies also serve as a valid and experimentally easier-to-handle model for the evolution of HIV from SIV. From these investigations of felids and their immunodeficiency viruses, we know that lentiviruses of lion (FIV-Ple) and puma (FIV-Pco) differentially replicate in activated PBMC of cats: FIV-Ple shows spreading replication, but FIV-Pco did not replicate in cat PBMC (50). Subsequently, the FIV-Pco isolate from experimentally infected cats contained extensive G-to-A mutations reminiscent of A3 activity (41). It was also described that pumas support the replication of FIV from bobcats that are frequently transmitted between these two species (12). In the cases where FIVs do replicate in heterologous species or PBMC, one can assume that the Vif protein can counteract the A3s of the novel, heterologous hosts. Alternatively, but less likely, it is possible that certain A3s are not active against some of the heterologous FIVs.

Based on our experiments, it appears that most of the diverse felid A3s of the domestic cat and big cats (lion, tiger, puma, and lynx) are probably not major determinants to prevent cross-species transmission of FIV of domestic cats to these closely related animal species. The A3Z2c-Z3 protein of tiger and the A3Z2-Z3 proteins containing amino acids of the polymorphic exon 4 of some cat breeds were found to be moderately resistance to the Vif of domestic cat FIV. For the tiger A3 we showed that low levels of cat Vif insufficiently counteracted its antiviral activity and regular Vif levels mostly inhibited this A3. Further experiments analyzing spreading FIV replications will be necessary to learn more about the inhibitory impact of these moderately resistant A3s during a longer observation period. We predict that FIV would quickly adapt its Vif protein to a complete A3 counteraction after a few rounds of replication in cells expressing, e.g., tiger A3Z2-Z3 protein. These assumptions are in fact supported by the ease with which FIV of the domestic cats infects other *Felidae*. A contributing factor may be that restriction of FIV replication and interspecies transmission by TRIM5 proteins is lacking in *Felidae* due to an in-frame stop mutation in the TRIM5 gene (29). However, other feline retroviruses and nonretroviruses might be more sensitive to the tested felid A3s in a species-specific manner, possibly explaining the positive selection on the felid A3 genes and the high prevalence of nonsilent mutations in the A3Z2 genes in different cat breeding lines (31). Polymorphic A3 genes are found also in humans, horses, and mice and show differential antiviral activities (1, 14, 36, 37, 58). Experiments with wt and *vif* FIVs of other felids will be required to ultimately demonstrate whether the FIV of the domestic cat is unique or whether many FIVs have the capacity to counteract heterologous felid A3s. The monophyly of the FIV strains of the diverse *Felidae* is a strong argument against frequent cross-species transmissions and, beside A3, other cellular and noncellular factors such as contact rates between different species likely determine or even limit interspecies FIV spread (51).

However, independent of whether feline A3s restrict FIV or not, the Vif_{FIV} protein recognizes diverse feline A3 proteins and induces their depletion (FcaA3Z3 versus FcaA3Z2 proteins). Due to this broad reactivity, Vif_{FIV} has the capacity to counteract also engineered two-domain A3Z2-Z3s that carried only one domain of feline origin (Fig. 4B and C). It is currently unclear whether feline A3Z2 and -Z3 proteins share a Vif interaction domain, whether Vif interacts independently with the Z2 and Z3 domains or whether the A3-Vif interaction in a single molecule of FcaA3Z2-Z3 happens with both Z-domains simultaneously or preferentially only with one domain. For the virus, it is an obvious advantage that Vif interacts with both Z domains. As a disadvantage for the feline host, the feline A3Z2-Z3 proteins need to evolve at two regions at the same time to escape the Vif-induced degradation. In contrast to FIV, the HIV-1 Vif protein binds only a single Z domain in the human A3F (HsaA3Z2e-Z2f) and A3G (HsaA3Z2g-Z1c): Z2f or the Z2g, respectively (17, 42, 56, 57). It is hard to guess whether the interaction of $Vif_{HIV\text{-}1}$ with only a single Z domain is advantageous for either the virus or the host, and we do not know why Vif of FIV appears to interact with both Z domains. The interaction of Vif_{FIV} with A3Z2s may be a relict of an FIV ancestor virus that was refractory also to these A3 types and had to evolve a Vif protein that targets also Z2 proteins. In this model, modern FIVs preserved the "ancient" function to interact with A3Z2. Alternatively, the limited reagents used in the present study might not properly reflect the FIV-cat interaction. Here, we tested reporter vectors based on the domestic cat strains FIV-PPR (40) and FIV-34TF10 (47). We cannot exclude that wt or Δ*vif* variants of other FIV strains, e.g., from other feline hosts, are more restricted by feline A3Z2 proteins than these two FIV isolates. Moreover, cats might carry thus far unidentified A3Z2 alleles that show a stronger inhibitory activity than the described Z2 proteins.

Because cats are resistant to HIV-1, we wanted to understand how many genetic changes in HIV-1 or cats would be required to circumvent this species block. We recently found that the feline CD4 receptor does not support HIV-1 infection, and we described the feline A3 proteins as restriction factors for HIV-1 (33). In order to prevent HIV-1 restriction by feline A3s, we replaced the *vif* gene of HIV-1 by *vif* of FIV. This HIV-1vif_{FIV} replicated in nonpermissive, feline A3-expressing CrFK.CD4.CCR5 cells to levels of wt HIV-1 replication in the permissive human HOS.CD4.CCR5 cells. Since the *Felidae*, including the domestic cat, do not encode a functional copy of the TRIM5 gene (29), cats likely will not exhibit a TRIM5-like restriction against HIV-1. Other limitations and restrictions for HIV-1 in cats might exist but, together, these results are encouraging to strengthen research on an animal model for HIV-1 based on the domestic cat.

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SUMMARY

APOBEC3 (apolipoprotein B mRNA editing catalytic polypeptide 3 [A3]) proteins are an element of the intrinsic immunity of modern mammalia against retroviruses. The evolution of the A3 genes is characterized by an adaptive selection and a taxon-specific expansion and/or extinction. All A3 genes consist either of one zinc (Z)-coordinating domain (Z1, Z2 or Z3) or two Z-domains (Z2-Z1, Z2-Z2, Z2-Z3). While humans carry seven A3 genes, *Equus caballus* carries six and *Felis catus* four A3 genes.

The genome of *Equus caballus* carries two Z1, two Z2-Z2, one Z2 and one Z3 A3 gene. Some of the equine A3 are able to restrict the *Equine infectious anaemia virus* (EIAV). Equine macrophages, which are the natural target cells for EIAV, express only a limited repertoire of the A3 genes. In addition, the transcriptional level of the anti-EIAV A3 mRNAs was significantly lower in macrophages than in equine peripheral blood mononuclear cells. Equine A3 proteins hyper-mutated EIAV genomes supporting their predicted function. In contrast to all other extant lentiviruses, EIAV does not encode a *vif* gene. Other EIAV-specific genes as *dUTPase* and *S2*, whose relevance for the viral replication in previous studies was only insufficiently characterized, did not influence the inhibitory effect of the equine A3 proteins. Thus, EIAV does not have an antagonist against its species-own A3 proteins. These findings indicate that lentiviral replication can occur independent from a *vif* gene, which likely developed later in the evolution of the lentiviruses.

Felis catus and of other *Felidae* encode A3 genes of the type Z2 and Z3. Beside one-domain molecules, also two-domain encoding read-through transcripts with two Vif interaction sites were detectable, that restricted different feline retroviruses. FIV (*Feline immunodeficiency virus*) encodes a Vif protein that counteracts the inhibitory effect of the feline A3 proteins. Despite a high genetic diversity in felid A3 genes, no strong resistance could be shown against the neutralizing activity of Vif of the domestic cat FIV. Non-feline A3 proteins restricted FIV independently of Vif, which proves that the interaction between A3 and Vif proteins is species-specific and thus limits interspecies virus transmission.

Species-specificity was also demonstrated for the interaction of feline A3 and the Vif protein of HIV-1. Feline cells that expressed human entry receptors were permissive for the transduction with a HIV-1 reporter virus but the virus was not able to spread in these cells. HIV replication in feline cells is strongly inhibited by the feline A3 proteins. In order to overcome this restriction, the *vif* gene of HIV-1 was replaced by vif_{FIV}. This chimeric HIV-1 showed spreading replication in feline cells expressing human receptors. Altogether, these findings implicate the importance of a novel animal model for HIV-1 based on *Felis catus*.

ZUSAMMENFASSUNG

APOBEC3 (*apolipoprotein B mRNA-editing catalytic polypeptide 3* [A3]) Proteine sind ein Element der intrinsischen Immunität heutiger Mammalia gegenüber Retroviren. Die Evolution der A3 Gene zeichnet sich durch eine adaptive Selektion und eine taxonspezifische Expansion bzw. Extinktion aus. Alle A3 Gene bestehen entweder aus einer Zink (Z)-koordinierenden Domäne (Z1, Z2 oder Z3) oder zwei Z-Domänen (Z2-Z1, Z2-Z2, Z2-Z3). Während bei den *Hominoidea* sieben A3 Gene identifiziert werden konnten, waren es bei *Equus caballus* sechs und bei *Felis catus* vier.

Im Genom von *Equus caballus* konnten zwei Z1, drei Z2 und ein Z3 A3 Gen detektiert werden. Einige der equinen A3 Proteine sind in der Lage *Equine Infectious Anemia Virus* (EIAV) zu restringieren. Equine Makrophagen, die dem equinen Lentivirus EIAV als natürliche Zielzellen dienen, exprimieren nur ein limitiertes Repertoire an A3 Genen. Das Transkriptionslevel der antiviralen A3 mRNA war in den Makrophagen signifikant kleiner als in den analysierten *Peripheral Blood Mononuclear Cells*. In ihrer Funktion als Cytosin Deaminasen verursachen equine A3 Proteine hypermutierte EIAV-Genome. Im Unterschied zu allen anderen existierenden Lentiviren ist das Vif Protein nicht im EIAV-Genom kodiert. Andere EIAV-spezifische Gene wie *dUTPASE* und *S2*, deren Bedeutung für die Virusreplikation in vorhergehenden Studien nur unzureichend nachgewiesen werden konnten, zeigten keinen Einfluss auf den inhibitorischen Effekt der equinen A3 Proteine. Somit fehlt EIAV ein Antagonist, der aktiv der A3 Restriktion entgegenwirkt. Dies deutet daraufhin, dass lentivirale Replikation unabhängig von einem *vif* Gen, das erst später in der Evolution der Lentiviren entstanden ist, erfolgen kann.

Im *Felis catus* Genom und anderen Vertretern der Felidae wurden ausschließlich A3 Gene der Nomenklatur Z2 und Z3 detektiert. Neben Ein-Domänen-Molekülen wurden auch durchlesetranskript-kodierte Proteine mit zwei Vif-Interaktionsseiten nachgewiesen, die das Potential besitzen, unterschiedliche feline Retroviren zu inhibieren. FIV (*Feline Immunodeficiency Virus*) kodiert für ein Vif Protein, dass der antiviralen Wirkung der felinen A3 Proteine entgegenwirkt. Trotz der gewichtigen genetischen Diversität der A3 Gene innerhalb der *Felidae* konnte keine Resistenz gegen die neutralisierende Aktivität des Vif Proteins gezeigt werden. Nicht-felide A3 Proteine restringieren FIV unabhängig von Vif, was beweist, dass die Interaktion zwischen A3 und Vif Proteinen spezies-spezifisch ist und dadurch eine interspezifische Virustransmission limitiert wird.

Die Spezies-Spezifität wurde ebenfalls für die Interaktion der felinen A3 Proteine mit dem Vif Protein von HIV-1 nachgewiesen. Feline Zellen, die humane Rezeptoren exprimieren, waren für die Transduktion mit einem HIV-1 Reportervirus permissiv, jedoch war das Virus nicht in der Lage, in diesen Zellen zu replizieren. Dieser zytoplasmatische Block lässt sich auf einen starken inhibitorischen Effekt der A3 Proteine zurückführen. Um diese Restriktion zu umgehen, wurde das *vif* Gen von HIV-1 durch *vif*$_{FIV}$ ausgetauscht. Das chimäre HIV-1 zeigte eine sich ausbreitende Replikation in felinen Zellen, die humane Rezeptoren exprimieren. Diese Ergebnisse sind von besonderer Bedeutung für ein auf *Felis catus* basierendes Tiermodell für HIV.

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Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation selbstständig und nur mit den angegebenen Hilfsmitteln angefertigt habe. Ich habe bisher noch keinen Promotionsversuch unternommen.

Jörg Zielonka
Düsseldorf, den 30. September 2010