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 (TRIM5α) and apolipoprotein B mRNA-editing enzyme-catalytic polypeptide 3 (APOBEC3 [A3]) (for a review, see reference 92). With respect to TRIM5α proteins, both human and nonhuman primate TRIM5α orthologues can restrict infection by EIAV (26, 72). The activity of equine TRIM5α 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 1 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 saker 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 Zφ 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 eqA3s 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 CFSK (ATCC CCL-94); and equine macrophage-like cells EML-3C (20)) were maintained in Dulbecco’s high-glucose medium (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 µg/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 at 0.3 million cells/ml in RPMI 1640 (Biochrom, Berlin, Germany) containing 10% fetal bovine serum, 5 × 10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, and 100 units of 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 medium with 5% 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 2000 (Invitrogen, Karlsruhe, Germany) according to 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 pONY8.1 (69), a self-inactivating vector variant of pONY8.0 (53) coding for enhanced green fluorescent protein and a deleted env region. The epg 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 pSI_{luc3} (where agm indicates the African green monkey SIV host) (49) as a template and the primers Eluc.fw (5’-CTCCTCGGGCTCCACGCTATGGAAGGACGCAA-3’) and NotI restriction site is underlined; where present, fw indicates forward and ri indicates reverse) and Eluc.rv (5’-AGGCGCGGCTTTACACGGCGATCTTT-3’; NotI restriction site is underlined), subcloned in pJET1.2/blunt (Fermentas, St. Leon-Rot, Germany), sequence verified, and recloned into pONYS.1 using SacII and NotI restriction sites. The procedure used to generate pONY3.1ΔDU results in an in-frame deletion of 270 bp in the predicted dUTPase (DUS) coding region (modified after Threadgill et al. [90]). By using pONY3.1 template DNA and the primer pair EIAV1.fw (5’-GGCGGCCGCGCTGGCAGACCACAGGAGGCT-3’) and EIAV-dUrv (5’-TTAATGTCCCTGACCTTACATCTT-3’) and the pair EIAV2.fw (5’-GGCGCGGCGCTGGCAGACCACAGGAGGCT-3’) and EIAV-dUrv (5’-TGGCTGGGCACTTAATCAGACATTGAG-3’), the pair EIAV-dUrv (5’-TGGCTGGGCACTTAATCAGACATTGAG-3’). SIV reporter viruses were produced via transfection of 293T cells with pSIV_{luc3} (where agm indicates a SIV-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, FCM, EML-3C, or HOS cells were seeded at 2.0 × 10^6 cells/ml 1 day before transfection in 96-well plates and then infected with reporter virus stocks normalized for reverse transcriptase. Firefly luciferase activity was measured 3 days post-transfection with a SteadyHit HTS reporter gene assay system (PerkinElmer, Coburg, Germany), according to the manufacturer’s directions, on a Berthold MicroLumat Plus luminometer. To generate recombinant AAV-2, 293 cells were transfected with the luciferase reporter plasmid pAAV-luc (51), the rrev-reversion encoding pAAV-RC (51, 93), and helper helper plasmid pAAV-2 (51) according to the manufacturer’s instructions. Recombinant AAV-2 was produced for infection of 293T 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.** A3s are expressed as carboxy-terminal (CT) hemagglutinin (HA)-tagged recombinant (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 muA3-Z2) were provided by N. R. Landau (49). Human A3B, -C, -F, and -H (A3Z2a-Z2f, A3Z2c-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 Ralph Tonjes (GenBank accession no. EU571587; 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 eqAP0BEC3Z1b (eqA3Z1b), the primers were E-AP0-37 (5’-TTTGTTGAAACACCTGTTGCAGATGACCTTC-3’) and E-APO3Cd-XhoI.rv (5’-GGCGCGCGCTGGCAGACCACAGGAGGCT-3’). Expression plasmids were purified from 293T cells 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.

**Expression of viruses.** Firefly luciferase activity of viruses was quantified by using a Cavidi HS Lenti RT kit (Cavidi Tech, Uppsala, Sweden). For reporter virus infections, FCM, EML-3C, or HOS cells were seeded at 2.0 × 10^6 cells/ml 1 day before transfection in 96-well plates and then infected with reporter virus stocks normalized for reverse transcriptase. Firefly luciferase activity was measured 3 days post-transfection with a SteadyHit HTS reporter gene assay system (PerkinElmer, Coberg, Germany), according to the manufacturer’s directions, on a Berthold MicroLumat Plus luminometer. To generate recombinant AAV-2, 293 cells were transfected with the luciferase reporter plasmid pAAV-luc (51), the rrev-reversion encoding pAAV-RC (51, 93), and helper helper plasmid pAAV-2 (51) according to the manufacturer’s instructions. Recombinant AAV-2 was produced for infection of 293T 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. Recombinant AAV-2 was produced for infection of 293T 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.
23). A3F mice h3FAS1 (E67α), h3FAS2 (E251α), and h3FAS1/A2 (E67α/E251α) were a gift of N. R. Landau; the mutant A3G-E259Q was a gift of Barbara Vol. 83, 2009 RESTRICTION OF EIAV BY APOBEC3 7549.

**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 0.5% Triton X-100 in PBS for 10 min, blocked in 1% BSA for 1 h, and treated with anti-HA antibody (MMS-101P; Covance, Munich, Germany) and anti-nucleolin (ab27558; Abcam, Cambridge, United Kingdom) in a 1:1,000 dilution in blocking solution for 1 h. Donkey anti-mouse Alexa Fluor 488 (Involveto, Karlsruhe, Germany) and anti-rabbit Alexa Fluor 594 were used as secondary antibodies in a 1:300 dilution in blocking solution for 1 h. 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 40× objective on a Zeiss LSM 510 Meta laser scanning confocal microscope. Optical sections were acquired from 1-μm-thick layers.

**Phylogenetic analysis.** All A3 sequences were obtained from the databases in mammalian species within Laurasia that were retrieved for analysis, as follows: Sus scrofa, A3Z2 (NM_001097784); Bos taurus, A3Z1 (EU864543), A3Z2 (NM_001097785), and A3Z3 (DQ794746); Ovis aries, A3Z1 (EU864543), A3Z2 (NM_001097784), and A3Z3 (EU864544); Camisgames, A3Z1 (H11032); Felis catus, A3Z1 (NM_001112710), A3Z2 (AWB83272), A3Z3 (AWB83273), and A3Z4 (ABW83271); Lynx lynx, A3Z3 (EU007553) and A3Z1 isolate 1 (EU007556); Puma concolor, A3Z2 (EU007554); Panthera pardus, A3Z3 (EU007551) and A3Z2 (ABAB26301); Panthera leo, A3Z3 (U007549), A3Z2 isolate 7 (EU007543), and A3Z2 isolate 2 (EU007544); Panthera tigris, A3Z2 (EU007550), A3Z2 (AAAY9623), A3Z2 isolate 2 (EU006361), and A3Z2 isolate 3 (EU006362). 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) (86) using the GTR+I+G 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 = 8697.63571; 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-Hasegawa) test to compare the split support between the SH and the MP trees 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+I+G 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 on the relaxed clock combined chain, and its likelihood was calculated under ML framework with the GTR+I+G model of evolution. 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 sampling 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): eqA3Z2, ABAB26301; eqA3Z3, ABAB26302; eqA3Z1, ABAB26303; eqA3Z4, ABAB26304; eqA3Z5, ABAB26305.
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. 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 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 three 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 macrophage-derived 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 macrophages express ~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 A3A1; 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; 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 A3A1 (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, and AAV-2.** To examine the restriction activity of eqA3 proteins, VSV-G pseudotyped EIAV-luc and SIV-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 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

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**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.
EIAV and reduced the infectivity by approximately threefold each (Fig. 3A). Similarly, eqA3Z1b was weakly active against SIV. In contrast, SIVagm infectivity was inhibited 70-fold by eqA3Z2e, and eqA3Z1b reduced the infectivity of AAV-2 by 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 A3F2 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, eqA3Z2c-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-
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 A32Z2c-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 was detected, corresponding to their very weak antiviral activity 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 Δvif 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, 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).
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-1 Δvif (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. 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.
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 0.103 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 eqA3Z2c (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 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 Euarchonta 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 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 Δvif 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 anti-viral 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 apreadaptation, facilitating—even mechanistically—the gene fusion event. The diversification of genes opens the door to neo-functionalization (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-ZZ and A2-ZZ1 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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