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Novel and Dynamic Evolution of Equine Infectious Anemia Virus Genomic Quasispecies Associated with Sequential Disease Cycles in an Experimentally Infected Pony

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We have investigated the genetic evolution of three functionally distinct regions of the equine infectious anemia virus (EIAV) genome (*env*, *rev*, and long terminal repeat) during recurring febrile episodes in a pony experimentally infected with a well-characterized reference biological clone designated $EIAV_{PV}$. Viral populations present in the plasma of an $EIAV_{PV}$-infected pony during sequential febrile episodes (18, 34, 80, 106, and 337 days postinfection) were amplified from viral RNA, analyzed, and compared to the inoculated strain. The comparison of the viral quasispecies showed that the inoculated $EIAV_{PV}$ quasispecies were all represented during the first febrile episode, but entirely replaced at the time of the second febrile episode, and that new predominant quasispecies were associated with each subsequent cycle of disease. One of the more surprising results was the in vivo generation of large deletion (up to 15 amino acids) in the principal neutralizing domain (PND) of gp90 during the third febrile episode. This deletion did not alter the competence for in vitro replication as shown by the analysis of a *env* chimeric clone with a partially deleted PND and did not altered the fitness of the virus in vivo, since this partially deleted envelope became the major population during the fourth febrile episode. Finally, we showed that the amino acid mutations were not randomly distributed but delineated eight variables regions, V1 to V8, with V3 containing the PND region. These studies provide the first detailed description of the evolution of EIAV genomic quasispecies during persistent infection and reveal new insights into the genetics and potential mechanisms of lentivirus genomic variation.

Infection of horses with equine infectious anemia virus (EIAV), a member of the lentivirus subfamily of retroviruses, has served as a model for immune-mediated antigenic variation. EIAV is unique among lentiviruses in that it causes a dynamic and relatively defined course of infection and recurring disease in infected animals. We have developed a pony model of nonlethal infection with a pathogenic stock of virus ($EIAV_{PV}$) that results in three stages of disease progression: acute, chronic, and inapparent (reviewed in reference 25). Typically, the first febrile episode defined as the acute stage occurs by about 3 weeks postinfection. Then, the ponies enter the chronic stage and experience 6 to 8 months of recurring cycles of viremia and disease (fever, diarrhea, anemia, and thrombocytopenia) at irregular intervals separated by weeks or months. Approximately 8 to 12 months postinfection, the ponies enter an inapparent stage in which they are clinically quiescent, although they remain infected for life. It has been demonstrated that each disease episode is associated with a novel predominant antigenic variant (18, 24). Biochemical (24, 40) and immunological (16, 31) studies of the predominant EIAV viral population recovered during the disease demonstrate an accumulation of mutations mainly in the gp90 surface (SU) and gp45 transmembrane (TM) glycoproteins, suggesting that the recurrence of the disease is associated with the emergence of new viruses that temporarily escape established immune surveillance.

Lentiviruses are among the most rapidly evolving genomes (25, 26). Low fidelity of the lentiviral reverse transcriptase, which lacks proofreading activity (34, 36), and the recombination of genomes within coinfected cells (37, 39, 49) may contribute to an elevated mutation rate. Lentiviruses are defined as viral populations of closely related genomes or quasispecies (10, 14). However, the role played by the genetic variability of lentiviruses in the development of the disease is not clearly established. While infections with lentiviruses such as human immunodeficiency virus type 1 (HIV-1) usually result in a slow progressive development of disease over many years, the rapid progression and the clear demarcation of the disease in EIAV-infected animals offer a unique model with which to analyze the link between the genetic variation of a lentiviral genome and the development of clinical symptoms.

To date, genetic variation studies of EIAV have analyzed only proviral DNA from viral strains isolated by cell culture from plasma of experimentally infected horses, raising the possibility of an in vitro selection of viral strains. There has been no analyses of plasma viral RNA quasispecies present during the recurring cycles of disease characteristic of chronic equine infectious anemia (EIA). In addition, the limited amount of EIAV genomic sequence information has precluded a detailed mapping of conserved and variable segments of the viral envelope. Thus, these limitations have prevented a realization of the potential utility of the EIAV system as a model for examining the nature and role of genomic and antigenic variations in maintaining viral persistence and for the development of disease.

To better characterize the viral quasispecies associated with the recurring febrile episodes, we have now analyzed the genetic evolution of the *env*, *rev*, and long terminal repeat (LTR) segments of the EIAV genome in a pony infected with the

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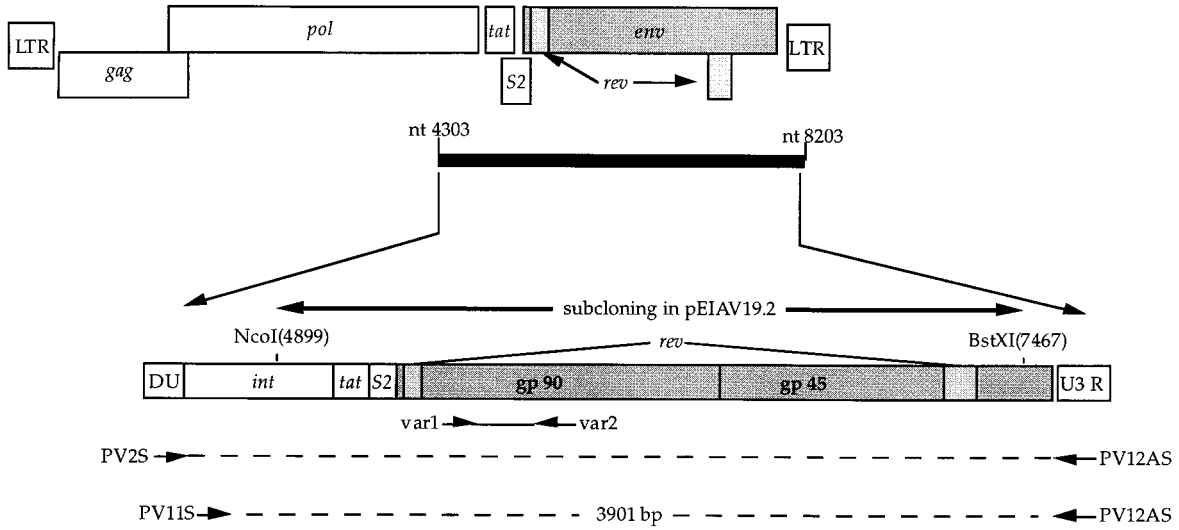


FIG. 1. RT-PCR, sequencing, and strategies for subcloning of the 3′ half of EIAV genome. The numbers indicated on the EIAV genome correspond to the reported pEIAV19-2 sequence (32 [GenBank accession no. U01866]). The RT reaction from plasma RNA was performed with the EIAV-specific primer PV12AS. For plasma RNA obtained during the first (564I), second (564II), third (564III), and fourth (564IV) febrile episodes, the cDNA was amplified by using a seminested PCR protocol, first with primers PV2S (sense) and PV12AS (antisense) and then with primers PV11S (sense) and PV12AS (antisense). The resulting fragment (about 3,900 bp) contained the end of the *pol* gene, the *env* gene, *tat*, S2, *rev*, and the U3-R region of the LTR. The cDNA obtained during the fifth episode (564V) was amplified by using primers Var1 (sense) and Var2 (antisense). The resulting amplified product was about 850 bp long. The *Nco*I/*Bst*XI fragment of clone 564IV.3 was exchanged in the nonpathogenic molecular clone pEIAV19-2, resulting in the *env* chimeric clone 19/564IV.3. nt, nucleotide; DU, dUTPase.

pathogenic stock of virus ($EIAV_{PV}$) during the initial episode (18 days postinoculation) and the subsequent four febrile episodes (34, 80, 106, and 337 days postinoculation) by reverse transcription (RT)-PCR amplification and sequencing of the plasma viral RNA. In parallel, the neutralizing antibody response against $EIAV_{PV}$ was monitored. The results of these studies reveal for the first time dynamic patterns of EIAV quasispecies variation that provide novel insights into the kinetics and mechanisms of lentivirus genomic variation and its role in viral persistence.

MATERIALS AND METHODS

Experimental infections, clinical evaluation, and sample collection. Pony 564 was experimentally inoculated intravenously with 10^3 50% tissue culture infective doses ($TCID_{50}$) of the pathogenic strain $EIAV_{PV}$ (38). The clinical and immune responses in this experimentally infected pony have been extensively described recently (12). Rectal temperatures and clinical status were recorded daily. Whole-blood samples were fractionated for enumeration of platelets (Unopette microcollection system; Becton Dickinson, Rutherford, N.J.). Samples of serum were collected at days 0, 7, 14, 21, 28, 35, 42, 49, 56, 70, 84, 119, 182, 238, 301, 304, and 364 and during each febrile episode and analyzed in a neutralization test as described below. Plasma samples were collected during each febrile episode (rectal temperature, >39°C; platelet number, <100,000/μl of whole blood) and stored at −80°C until RNA extraction.

Neutralization antibody assay. The development of serum neutralizing antibodies during the course of the infection was assessed in an indirect cell-based infectious center enzyme-linked immunosorbent assay (12). Sera were heat inactivated for 1 h at 56°C before use in assay to inactivate infectious virus and labile serum proteins. Briefly, 10^5 fetal equine kidney (FEK) cells were added into each well of a 24-well tissue culture plate and cultured at 37°C for 18 to 24 h. Twofold serial dilutions of each serum sample were incubated with 100 infectious centers of $EIAV_{PV}$ for 1 h at 37°C. An overlay of 0.8% carboxymethylcellulose was added to the infected cell culture to prevent the spread of the virus throughout the entire culture and localize virus infection to defined centers. The infected cultures were further incubated at 37°C for 9 days. After fixation with 3.7% of formaldehyde and permeabilization with 1% Triton X-100, infected cells were detected by conducting a cell-based enzyme-linked immunosorbent assay. The primary antibody used was a 1:200 dilution of a reference immune serum from an EIAV-infected horse (Lady) (24). The secondary antibody was a 1:3,000 dilution of an affinity-purified, horseradish peroxidase-conjugated, goat anti-horse immunoglobulin G (United States Biochemical Corporation). The peroxidase substrate used to visualize EIAV infectious centers was 3-amino-9-ethylcarbazole (Sigma) in a sodium acetate buffer (pH 5.5) supplemented with H_2O_2. The enzymatic reaction was terminated with water, and the visually apparent foci were enumerated with a dissecting microscope. The 50th percentile reciprocal neutralization titers of each serum sample were determined by linear regression analyses of the $\log_{10}$ reciprocal dilution versus the number of apparent infectious centers.

RNA purification and RT-PCR. Viral RNA was extracted from plasma samples by Trizol (Gibco BRL) treatment of virus pellets obtained by centrifugation at 120,000 × *g* at 4°C for 45 min. Purified viral RNA (2 to 5 μl) was reverse transcribed with the Superscript PreAmplification system (Gibco BRL) as specified by the manufacturer, using the EIAV-specific primer PV12AS (Fig. 1). Amplification of the specific fragment from plasma RNA samples obtained during febrile episodes I to IV was performed by using Elongase mix (*Taq*/*Pyrococcus* species GB-D DNA polymerase mixture; Gibco BRL) with a mixture of 60 mM Tris SO_4 (pH 9.1), 18 mM $(NH_4)_2SO_4$, 1.5 mM $MgSO_4$, 0.01 mM each deoxynucleoside triphosphate, 0.4 μM each primer, 2 μl of Elongase mix, and 3 μl of cDNA in a final volume of 50 μl. A initial PCR was performed with primers PV2S (sense) and PV12AS (antisense) (Table 1). A second seminested PCR was then carried out with primers PV11S (sense) and PV12AS and 3 μl of the first PCR product. The following conditions were used: 1 min at 95°C for the initial denaturation step; 20 s at 95°C, 20 s at 50°C, and 6 min at 68°C for 35 cycles; 10 min at 68°C for one cycle. For plasma RNA of episode V, a smaller fragment corresponding to the variable region of gp90 was amplified with Var1 (sense) and Var2 (antisense) primers (Table 1) as previously described (22).

Cloning of RT-PCR products. Several independent RT-PCR products (at least two independent RT reactions and three or four independent PCRs) were generated from plasma samples taken during each febrile episode, purified by using the Wizard PCR Preps DNA purification system (Promega, Madison, Wis.), and cloned. Due to the highly unstable nature of EIAV *env* sequences when associated with high-copy-number plasmids in transformed *Escherichia coli* (6), the 3,901-bp fragments obtained from $EIAV_{PV}$ stock and the plasma samples during febrile episodes I to IV were cloned into the low-copy-number vector pLG338, using the *Eco*RI and *Kpn*I sites located in the sense (PV11S) and antisense (PV12AS) primers, respectively. The RT-PCR products generated from episode V were cloned into the pGEM5Zf(+) T-A vector (Promega). The ligation products were used to transform competent *E. coli* DH5α. The clones were screened by a standard colony hybridization technique (42), using a ^{32}P-labeled EIAV probe. The positive clones were then checked by restriction enzyme digests for the proper size insert.

Sequencing of RT-PCR clones. Plasmid DNA was extracted and purified with a midiprep kit (Qiagen). The clones were automatically sequenced with a Taq Dye Deoxy Terminator Cycle Sequencer kit (Applied Biosystems), using internal EIAV primers (Table 1). DNA sequences were resolved with an ABI Prism 373 DNA sequencer (Applied Biosystems). The sequences were analyzed by using the Genetics Computer Group (Madison, Wis.) package analyses software (9).

To estimate the Elongase DNA polymerase error rate, 30 fg of a pEIAV19-2 (32 [GenBank accession no. U01866]) DNA was amplified by using PV2S and PV12AS, cloned, and sequenced as described above. The error was determined to be 0.015% (3 substitutions per 19,510 bp sequenced).

TABLE 1. EIAV-specific primers used for RT-PCR and sequencing

Name	Primer sequence[a] (5′→3′)	Location (nucleotides)	Used for:
PV2S	cggaattcCTCAGAGAGGGGATAAAGG	4276–4294	PCR
PV11S	ccggaattccggGTACAGGAGTATTCTGGGTAG	4303–4323	PCR
Var1	GTTCCTTCCCGGGGTGTAGACC	5692–5713	PCR
Var2	GAGGAGTTATATTGGTTAAAGCTTTGG	6544–6518	PCR
PV12AS	cggggtaccccgTGAGTAGAGAATTATATTTATTAC	8293–8270	PCR
S2	GTTATAAGGTTTGATATATGGGAT	5279–5302	Sequencing
S19	GGGCAAGATTGTTTTCACCAAAGTCC	4628–4652	Sequencing
S3	GCATATGGATAATAATACTGCTAC	5753–5776	Sequencing
S4	CTGCAATAATGAGCCAATAATCAG	6350–6373	Sequencing
S5	GAGGTAGAAAATAGTACTCTAAATGG	6780–6805	Sequencing
S6	CATCACAAACATGCATCGCGAGAAG	7317–7341	Sequencing
S7	TATGGGATTACGTGGACTCGCTGTTA	7754–7779	Sequencing
S10	TCAAGCATTTTTCTGATTATTTC	7840–7818	Sequencing
S11	ACTCCTCTGATTCTCCATTCCAGTCG	7458–7433	Sequencing
S12	TTCCATTTTGCTTACCCAGTCATCCC	7031–7006	Sequencing
S13	GGAGTTACGCCAGAAGTCTTTAC	6604–6582	Sequencing
S14	CGTCTCATTACACCCCTTAGGTATCC	6059–6034	Sequencing
S15	CCAAAGTATTCCTCCAGTAGAACCTG	5594–5569	Sequencing
S16	ATCCCATATATCAAACCTTATAAC	5302–5279	Sequencing

[a] The primers are located on the pEIAV19-2 complete sequence (32 [GenBank accession no. U01866]). The EIAV-specific sequences are indicated in uppercase; the added restriction enzyme (*Eco*RI for PV2S and PV11S; *Kpn*I for PV12AS) sites are indicated in lowercase.

Molecular cloning. The infectious, nonpathogenic molecular clone pEIAV19-2 (32 [GenBank accession no. U01866]) was used as a backbone for the generation of chimeric proviral clones by restriction enzyme fragment exchanges. The *Nco*I/*Bst*XI fragment (containing the end of the integrase region, the gp90 region, and part of the gp45 region) of clone p564IV.3 was used to replace the corresponding fragment of pEIAV19-2, resulting in the *env* chimeric clone 19.2/564IV.3 (Fig. 1). The replication of this clone carrying a partially deleted principal neutralizing domain (PND) was tested by transfection of 2×10^5 FEK cells with 1 μg of purified DNA, using 10 μl of Lipofectamine reagent (Gibco BRL). Virus production was assayed by weekly measurement of the reverse transcriptase activity in the supernatants of the transfected cells, using a micro-RT assay. Briefly, 10 μl of clarified supernatant was assayed in a 60-μl (final volume) reaction mixture containing 83.3 mM Tris-HCl, 10 mM KCl, 8.3 mM $MgCl_2$, 8.3 mM dithiothreitol, 0.083% Nonidet P-40, 8.3 mM EGTA, 18.3 μg of poly(rA) · poly$(dT)_{12-18}$ (Pharmacia Biotech, Piscataway, N.J.) per ml, and 1.5 μCi of [*methyl*-^{3}H]TTP (40 Ci/mmol; Amersham Life Sciences, Arlington Heights, Ill.). Following incubation at 37°C for 2 h, the entire sample was spotted onto a DE-81 filter disc (Whatman, Maidstone, England). Discs were dried under a heat lamp, washed three times in 1× SSC (15 mM NaCl, 1.5 mM sodium citrate) and once in 95% ethanol, and then counted in Ecolite scintillation fluid (ICN, Costa Mesa, Calif.).

Nucleotide sequence accession numbers. The sequences analyzed were submitted to GenBank and have been assigned accession no. AF005104 through AF005151.

RESULTS

Clinical and serological profiles of the $EIAV_{PV}$-infected pony 564. After inoculation with the $EIAV_{PV}$ pathogenic strain, pony 564 experienced five febrile episodes, at days 18 (564I), 34 (564II), 80 (564III), 106 (564IV), and 337 (564V) postinfection (Fig. 2A). The clinical EIA episodes were defined by rectal temperature above 39°C and by platelet count below 100,000/μl of whole blood. Infectious EIAV circulating in the plasma peaked in titer during each febrile episode (10^5, $10^{5.5}$, $10^{4.5}$, $10^{4.5}$, and $10^{4.5}$ $TCID_{50}$ during episodes I, II, III, IV, and V, respectively) and declined to undetectable levels after the fevers had subsided (reference 19 and unpublished data). Measurements of serum neutralization of the challenge strain in pony 564 indicated an interesting pattern in which detectable neutralizing antibodies appeared only after the third febrile episode (about 75 days postinfection), reached a maximum after the fourth febrile episode (at 110 days postinfection), and then declined to minimum levels during the quiescent period of the disease (Fig. 2B). After a quiescent period of 192 days, the unusual recrudescence of clinical EIAV at 337 days postinfection was associated with a rapid rise in the levels of neutralizing antibodies against $EIAV_{PV}$. The initial disappearance and surprising reemergence of measurable neutralizing antibodies in this infected pony suggested an unique genetic evolution of the viral quasispecies that warranted a detailed analyses.

Genetic variation during sequential disease cycles. To analyze the viral quasispecies associated with sequential febrile episodes, we monitored the genetic evolution of the *env* and *rev* genes and the LTR region by sequencing the viral plasma RNA obtained during each febrile episode and compared the sequences obtained to the sequences of the inoculum $EIAV_{PV}$ strain. The quasispecies present in the $EIAV_{PV}$ biological clone have been recently defined in a limited fragment (807 bp) of the *env* gene and the U3 region of the LTR (22). To extend the characterization of this strain, we sequenced the entire *env* gene and the LTR to encompass coding and regulatory segments of the viral genome.

The 3,901-bp amplification products contained part of the integrase, the *env* gene and the regions coding for the regulatory proteins S2, Tat, and Rev, and the LTR region (Fig. 1). The same fragment was amplified by RT-PCR from the plasma viral RNA obtained during the first (564I) through the fourth (564IV) febrile episode and from the $EIAV_{PV}$ biological clone. Independent RT-PCR products were cloned in the low-copy-number vector pLG338, using the *Eco*RI and *Kpn*I sites present in the primers. A smaller fragment of about 850 bp, corresponding to the previously described variable region of gp90, was amplified from the plasma viral RNA obtained during the fifth episode (564V). A total of 43 clones were sequenced from the ATG start codon of the *env* gene (nucleotide 4303) to the end of the R region of the LTR (nucleotide 8203): 10 clones for the first febrile episode (564I); 5 clones for the second episode (564II); 8 clones for the third episode (564III); 10 clones for the fourth episode (564IV); 10 clones for the fifth episode (564V); and 5 clones for the $EIAV_{PV}$ viral stock. The percentages of nucleotide divergence (0.09 to 2.6%) observed in vivo (Table 2) were always above the error rate determined

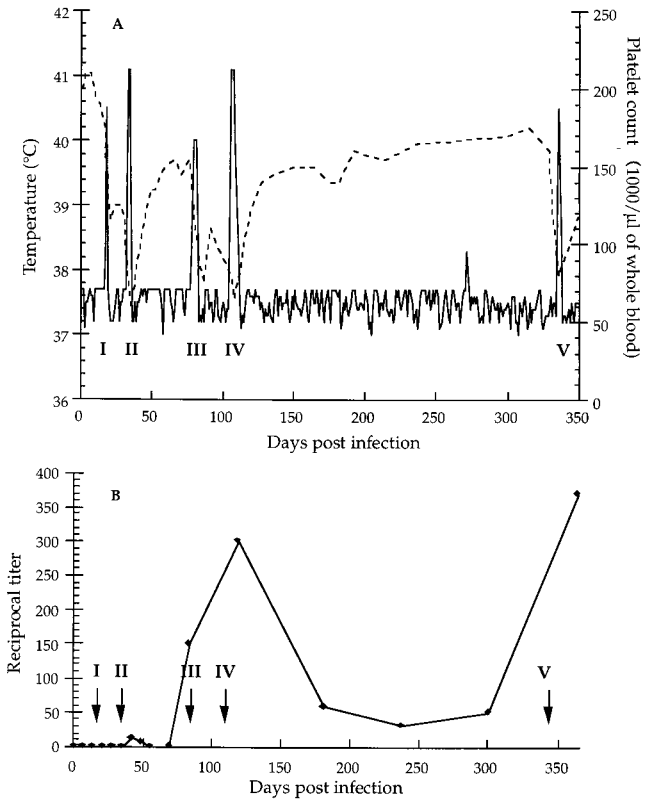


FIG. 2. Clinical course and neutralizing antibodies profiles in $EIAV_{PV}$-infected pony 564. (A) Clinical profile of pony 564. Pony 564 was experimentally infected with 10^3 $TCID_{50}$ of the biological clone $EIAV_{PV}$. Rectal temperature (solid line, primary *y* axis) and platelet count (dashed line, secondary *y* axis) were monitored for up to 350 days (*x* axis) after infection. Febrile episodes occurred at 18 (I), 34 (II), 80 (III), 106 (IV), and 337 (V) days postinfection and were defined by a rectal temperature above 39°C in conjunction with a reduction in the number of platelet below 100,000/μl of whole blood and other clinical symptoms. (B) $EIAV_{PV}$-specific neutralizing antibodies directed against the inoculated strain $EIAV_{PV}$. The 50% neutralizing antibody titers (*y* axis) were determined from serum samples obtained at 0, 7, 14, 21, 28, 35, 42, 49, 56, 70, 84, 119, 182, 238, 301, 304, and 364 days postinfection (*x* axis). The febrile episodes I through V are indicated by arrows.

for the PCR (0.015%), suggesting that most of the mutations occurred in vivo.

A detailed analysis of the genetic variation observed during sequential febrile episodes is described below.

Genetic variation in the U3-R region of the LTR. The sequences of the 3′ LTRs from the $EIAV_{PV}$ stock and the pony 564 plasma-derived samples were nearly identical (Table 2), with 0.09 to 0.43% nucleotide divergence from the $EIAV_{PV}$ stock in the second and fourth febrile episodes, respectively. None of the observed nucleotide divergence from the consensus sequences in the U3 region of the LTR of pony 564 were preexisting in clones obtained from the $EIAV_{PV}$ stock (Fig. 3A), suggesting that they appeared in vivo during the replication of the virus or represented a minor population in the $EIAV_{PV}$ stock. In the U3 region, the unique population U3a present in $EIAV_{PV}$ stock was progressively replaced by a more complex mixture and was no longer detected in the plasma by the fourth episode. As previously described (22), the U3 region of the LTR sequences (Fig. 3A) of $EIAV_{PV}$ and of the plasma-derived RNA lacked the second CAAT motif and the third *ets* motif shown to be present in the pathogenic Wyoming strain and suggested to be important for the viral pathogenesis (5) and for replication in macrophages (23). None of the mutations in the R region altered the transactivation responsive element (TAR) or the AATAAA hexamer. Some variations accumulated in the internal region of R, between the TAR element and the poly(A) site. The two populations (Ra and Rb) found in the $EIAV_{PV}$ stock were not replaced in vivo but persisted in association with in vivo-generated sequences until the fourth febrile episode, 106 days postinfection (Fig. 3B).

Genetic variation in *rev*. The third EIAV open reading frame (S3) has been shown to encode a regulatory protein (45), functionally analogous to the Rev protein described for other lentiviruses such as HIV-1 (7), visna-maedi virus (48), caprine arthritis encephalitis virus (41), or feline immunodeficiency virus (FIV) (33). As shown in Table 2, nucleotide and amino acid mutations slowly accumulated in the *rev* coding region. The analysis of the second exon of *rev* located at the 3′ end of the *env* gene during the successive febrile episodes showed an accumulation of mutations in the protein C terminus (Fig. 4). Of the 43 clones analyzed, 1 clone obtained from episode II presented a premature stop codon. The protein N terminus described as the activation domain of EIAV Rev (8) was perfectly conserved among the clones except for one clone that had an insertion of a lysine encoded by an AAA triplet. By the fourth episode, the major population present in the $EIAV_{PV}$ stock (REVa) had been replaced by a new quasispecies derived from REVa (Fig. 4).

Genetic variation in *env*. We next determined the nucleotide sequence encoding the surface (gp90) and transmembrane (gp45) glycoproteins of EIAV. Based on the consensus sequence of our $EIAV_{PV}$ stock, the total length of the *env* gene

TABLE 2. Mean percentage of variation[a] in gp90, gp45, *rev*, and LTR

Episode	Mean % variation						
	gp90		gp45		*rev*		LTR, nt
	nt[b]	aa[c]	nt	aa	nt	aa	
564I	0.12	0.2	0.15	0.14	0.32	0.54	0.19
564II	0.55	1.3	0.19	0.14	0.55	0.62	0.09
564III	0.64	1.5	0.4	0.6	0.63	1.4	0.29
564IV	0.7	1.8	0.4	1	0.75	1.7	0.43
564V	2.6	7.3	NS	NS	NS	NS	NS

[a] Determined by pairwise comparison of the 564 sequences and the $EIAV_{PV}$ consensus sequences. NS, not sequenced.
[b] Total number of nucleotide (nt) substitutions, with respect to the $EIAV_{PV}$ consensus sequence, divided by the total number of nucleotides sequenced, expressed as a percentage.
[c] Total number of deduced amino acid substitutions, with respect to the $EIAV_{PV}$ consensus sequence, divided by the total number of amino acids, expressed as a percentage.

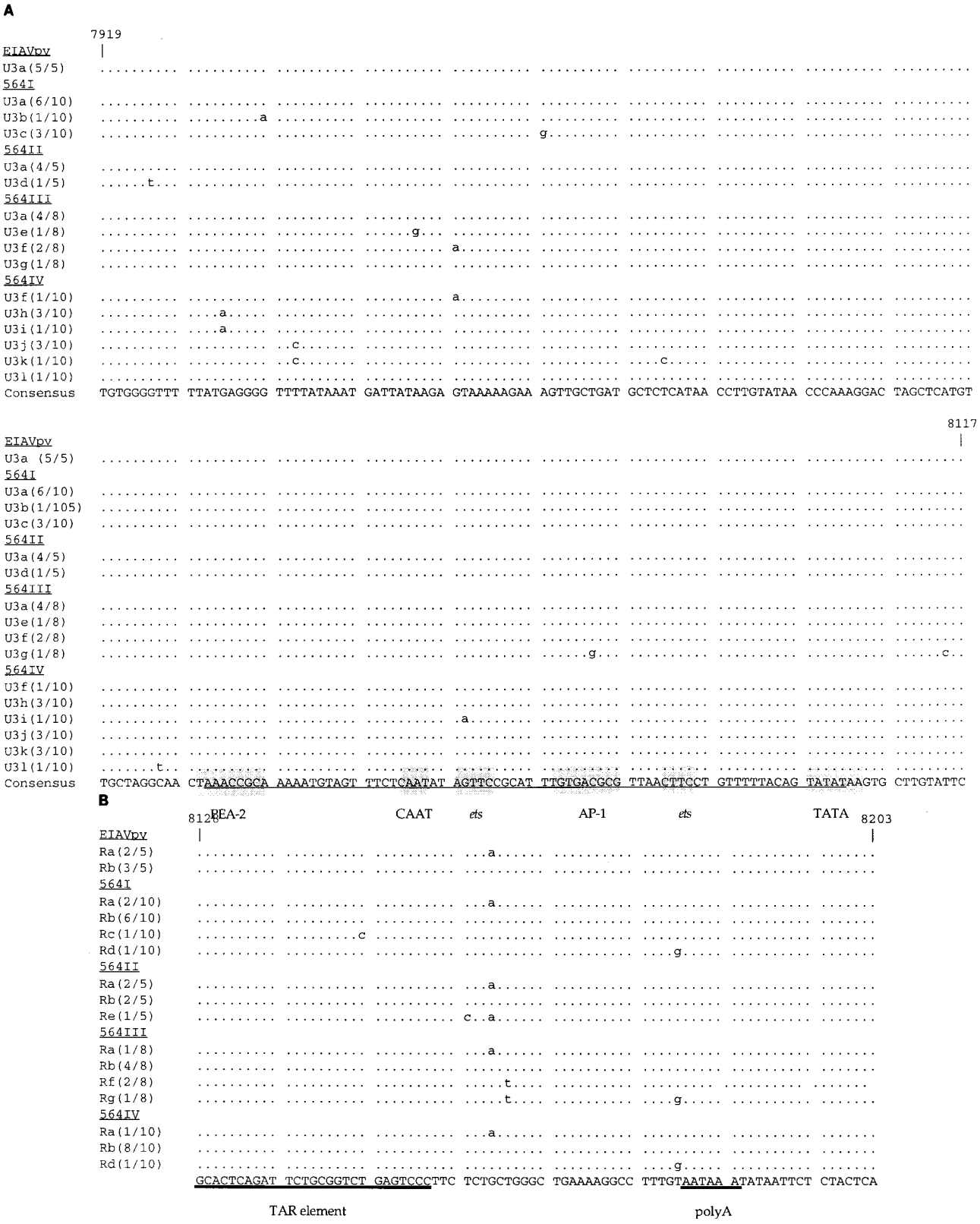


FIG. 3. Comparison of U3-R LTR sequences of $EIAV_{PV}$ and febrile episodes I to IV of $EIAV_{PV}$-infected pony 564. Sequences were aligned by using the Genetic Computer Group sequence analysis package (9) and numbered according to the pEIAV19-2 sequence (GenBank accession no. U01866). Only the nucleotides that differ from the nucleotide consensus sequence of the 43 clones are indicated. Dots indicate a nucleotide identical to the consensus sequence. For each sample, the different sequences are indicated on the left by the name of the sequence and the number of clones with a given sequence in parentheses. (A) Alignment of the U3 portion of the LTR. Potential *cis*-acting sequences (PEA-2, CAAT, *ets*, AP-1, and TATA) are boxed. The previously reported (5) hypervariable region is underlined. (B) Alignment of the R region of the LTR. The TAR element and the poly(A) hexamer (AATAAA) are underlined.

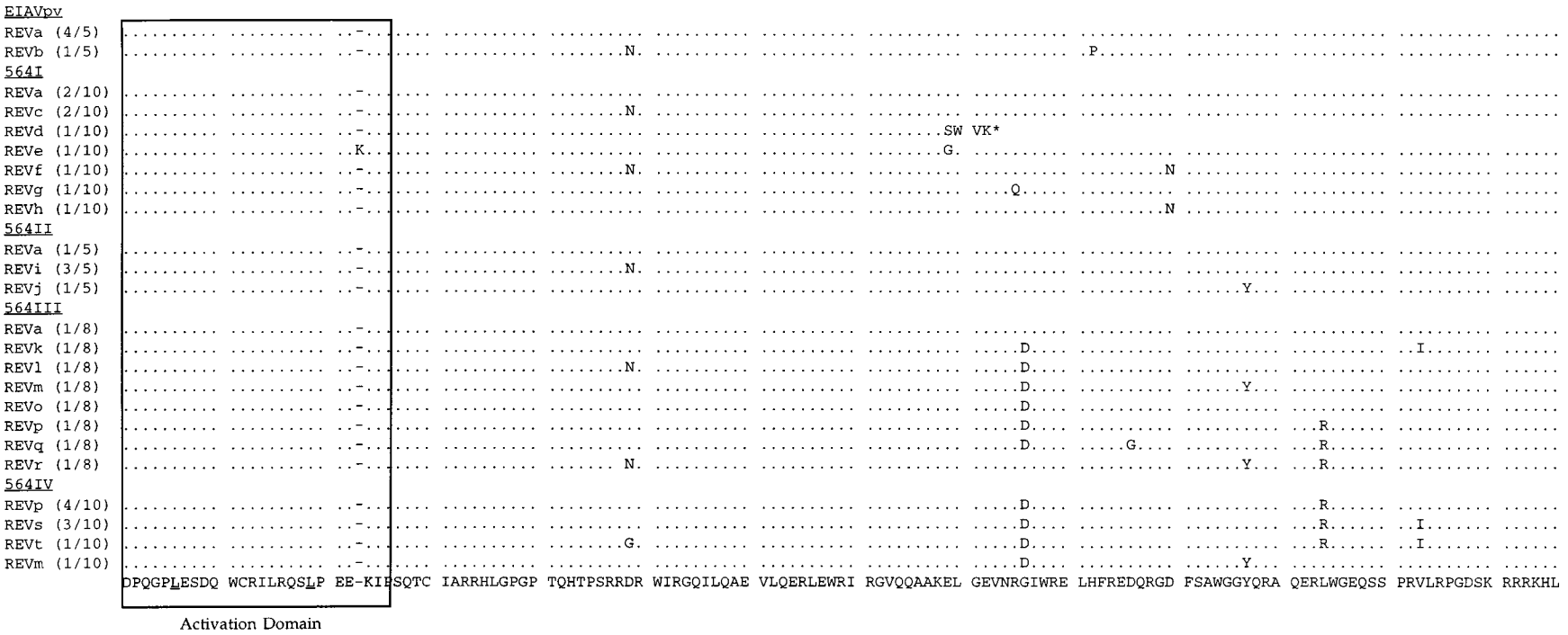


FIG. 4. Comparison of deduced amino acid sequences of the second exon of *rev*. Second exon of *rev* (nucleotides 7244 to 7651 of pEIAV19-2) located in the gp45 coding region of *env* was sequenced from the $EIAV_{PV}$ stock and plasma RNA 564I to 564IV. The deduced amino acid sequences were aligned by using the Genetic Computer Group sequence analysis package (9). Only the amino acids that differ from the amino acid consensus sequence are indicated. For each sample, the different sequences are indicated on the left by the name of the sequence and the number of clones with a given sequence in parentheses. A dot indicates a nucleotide identical to the consensus sequence; a dash indicates a deleted amino acid; an asterisk indicates a premature stop codon. The activation domain of Rev (8) is boxed, and the essential leucine residues are underlined.

is 2,577 nucleotides, with 1,332 nucleotides coding for gp90 (444 amino acids [aa]) and 1,245 nucleotides coding for gp45 (415 aa). Nucleotide and amino acid mutations accumulated predominantly in gp90 and to a lesser extent in gp45 (Table 2). The original $EIAV_{PV}$ gp90 quasispecies contained in the viral inoculum were present during the initial febrile episode at 18 days postinfection but were rapidly replaced by new quasispecies and were no longer detectable in the plasma by the time of the second febrile episode, 34 days postinfection (Fig. 5). These data demonstrate that the replicating quasispecies can be completely replaced in as little as 2 weeks between febrile episodes. In addition, analysis of viral quasispecies present during subsequent febrile episodes indicated the evolution of novel predominant viral RNA quasispecies associated with each disease episode. These observations are consistent with the model of sequential evolution and immune escape of EIAV antigenic variants that produce cycles of viremia and disease.

Variable amino acid positions were scored by the existence of at least two clones containing an amino acid residue that differs from the amino acid present in the consensus sequences. This analysis showed that 49 (11%) of the 444 aa of gp90, but only 5 (1.2%) of the 415 aa of gp45 (data not shown), were variable (Fig. 5). A variable region (aa 141 to 364) and a hypervariable region (HVR; aa 306 to 340) have been found in the gp90 of EIAV (30, 31). The percentages of variable amino acids were 15.6 and 17, respectively, in the variable region and the HVR.

EIAV gp90 is highly glycosylated, and based on the amino acid consensus sequence of $EIAV_{PV}$, 17 potential N-linked glycosylation sites (NX[S/T]) are present in gp90, compared with only 5 in gp45. Six (12%) of the 49 variable amino acids in gp90 involved a potential N-linked glycosylation site. Three new N-linked glycosylation sites were observed in some of the clones (Fig. 5). Five N-linked glycosylation sites are present in gp45. One of these sites involved a hypervariable amino acid residue at position 754 (Fig. 6). It has been experimentally demonstrated that all the potential N-linked glycosylation sites are modified in HIV-1 gp120 (20), and it seems reasonable to propose that most of these sites are used for EIAV. The glycosylation pattern of the EIAV envelope protein indicates that this modification may be a major determinant of envelope antigenic properties as proposed previously (29, 30, 40).

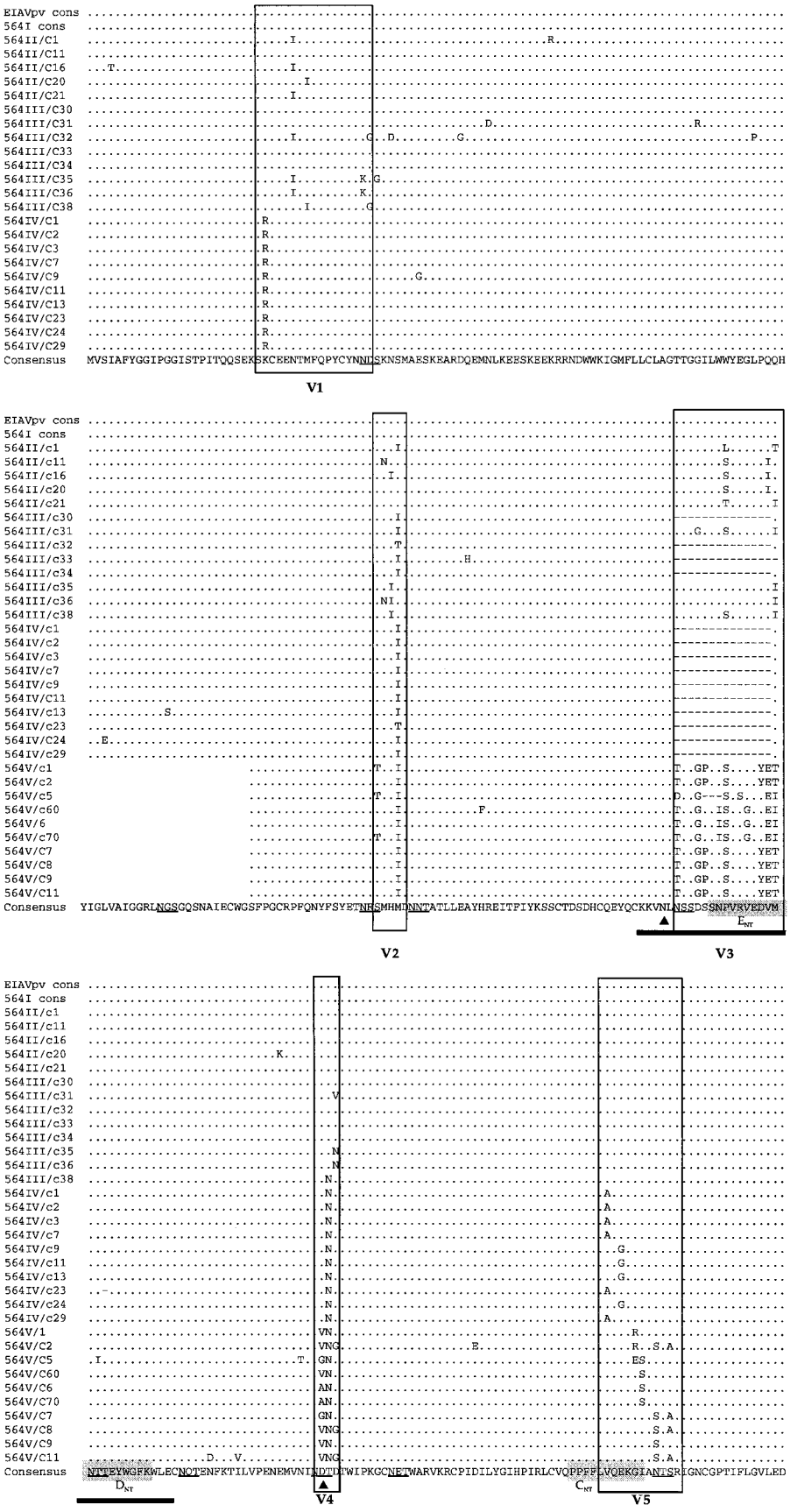
Despite the extent of amino acid replacement within gp90, the 19 cysteine residues present in the $EIAV_{PV}$ consensus sequence were perfectly conserved in the 43 analyzed clones. Conserved cysteine residues appear to perform an essential role in maintaining the required protein conformation. The odd number of cysteine residues conserved in EIAV gp90 indicates that not all of these residues are involved in disulfide bonding, yet all are essential for envelope protein structure and function.

In addition to modifications in envelope amino acid sequences, a length polymorphism was clearly visualized by resolution of RT-PCR products generated with primers Var1 and Var2 on ethidium bromide-stained agarose gel (Fig. 7). The single large (about 850-bp) DNA fragment present in episodes I and II became a mixture of large and small fragments (about 810 bp) in episode III and was completely replaced with the small fragment by episode IV. The fifth episode was associated with the reappearance of only the large fragment. Analysis of the nucleotide and amino acid sequences demonstrated that the length polymorphism observed was associated with deletions in the region previously described as the PND (3), located between two cysteine residues at positions 180 (C_{180}) and 213 (C_{213}) of gp90 (Fig. 5). Size variations were the results of a 9 (564V clone 5)- to a 45 (564IV clone 23)-nucleotide deletion resulting in a 3- to 15-aa deletion in the PND. A complete PND (34 aa) was found in all the clones obtained from $EIAV_{PV}$ stock, 564I or 564II (Fig. 5). During the third episode, half (four of eight) of the sequenced clones had a deletion of 14 aa in the PND. The quasispecies present in the plasma of pony 564 during the third episode appeared to be composed of two major populations in the V3 region. The coexistence of the two PND populations was also assessed by a PCR screening assay using primers Var1 and Var2 (Fig. 1). Due to the large deletion in the V3 region, the clones with the complete or the partially deleted PND were easily visualized on an ethidium bromide-stained agarose gel. Of the 43 tested clones, 26 (60%) had a deletion. By the fourth febrile episode, the replicating viral strains exclusively contained a PND deleted of 14 (9 of 10 clones) or 15 (1 of 10) amino acids. During the third and the fourth febrile episodes, the deletion completely removed E_{NT}, a sequence described as one of the principal neutralizing epitopes (3), and the downstream region. Surprisingly, the viral populations associated with the late recrudescence of the disease during the fifth febrile episode had a complete PND but with sequences clearly distinct from those of the PND present in the $EIAV_{PV}$ inoculum (Fig. 5).

The level of amino acid variation in gp45 was minimal. Only 5 of the 415 aa of gp45 were different from the consensus sequence in at least 2 of the 43 clones. Amino acid residues 568, 580, 726, and 754 were defined as hypervariable (Fig. 6).

Identification of variable and constant regions in EIAV gp90. The alignment of the deduced amino acid sequences (Fig. 5) demonstrated that the mutations in gp90 were not randomly distributed but were contained in defined variable regions. By a method similar to that described for the bovine lentivirus BIV (46), the variable regions of EIAV gp90 were defined as stretches of amino acids with more than 30% divergence from the amino acid consensus sequence. Only the amino acid residues for which mutations were observed in at least 2 of the 43 analyzed clones were used to define the variable regions. Based on the comparison of the deduced amino acid sequences of the 43 clones obtained from $EIAV_{PV}$ stock and plasma RNA of pony 564, we delineated eight variable regions in gp90 (Fig. 5 and 8): V1 (aa 26 to 42), V2 (aa 143 to 146), V3 (aa 186 to 201), V4 (aa 234 to 237), V5 (aa 275 to 284), V6 (aa 307 to 318), V7 (aa 366 to 377), and V8 (aa 393 to 401). Two conserved regions, C_N (aa 1 to 110) at the NH2 terminus and C_C (aa 370 to 445) at the COOH terminus, a variable region (aa 111 to 370) and an HVR (aa 306 to 336) have been previously found in gp90 (30). Our analysis showed that V1, V2 to V6, and V7 and V8 were in the C_N, variable, and C_C regions, respectively.

Replication of an *env* chimeric clone with a 14-aa-deleted PND. To test the replication of the virus with a partially deleted PND, we constructed a chimeric molecular clone by exchanging the *Nco*I/*Bst*XI fragment (Fig. 1) of clone 564IV.3 (carrying a 14-aa deletion in the PND) in our nonpathogenic molecular clone pEIAV19-2 (32). The resulting molecular clone 19.2/564IV.3 and the wild-type pEIAV19-2 were used to transfect FEK cells. The cell culture supernatants were assayed weekly for reverse transcriptase activity as a measure of virus production. The results indicated that the PND deletion in 19.2/564IV.3 clone did not cause a defect in the viral replication. Levels of virus production were equivalent in FEK cells transfected either with the parental or the chimeric clone (Fig. 9). Viruses produced after transfection had the deleted PND genotype, as confirmed by RT-PCR on purified viral RNA from the supernatant, and were infectious, as demonstrated by



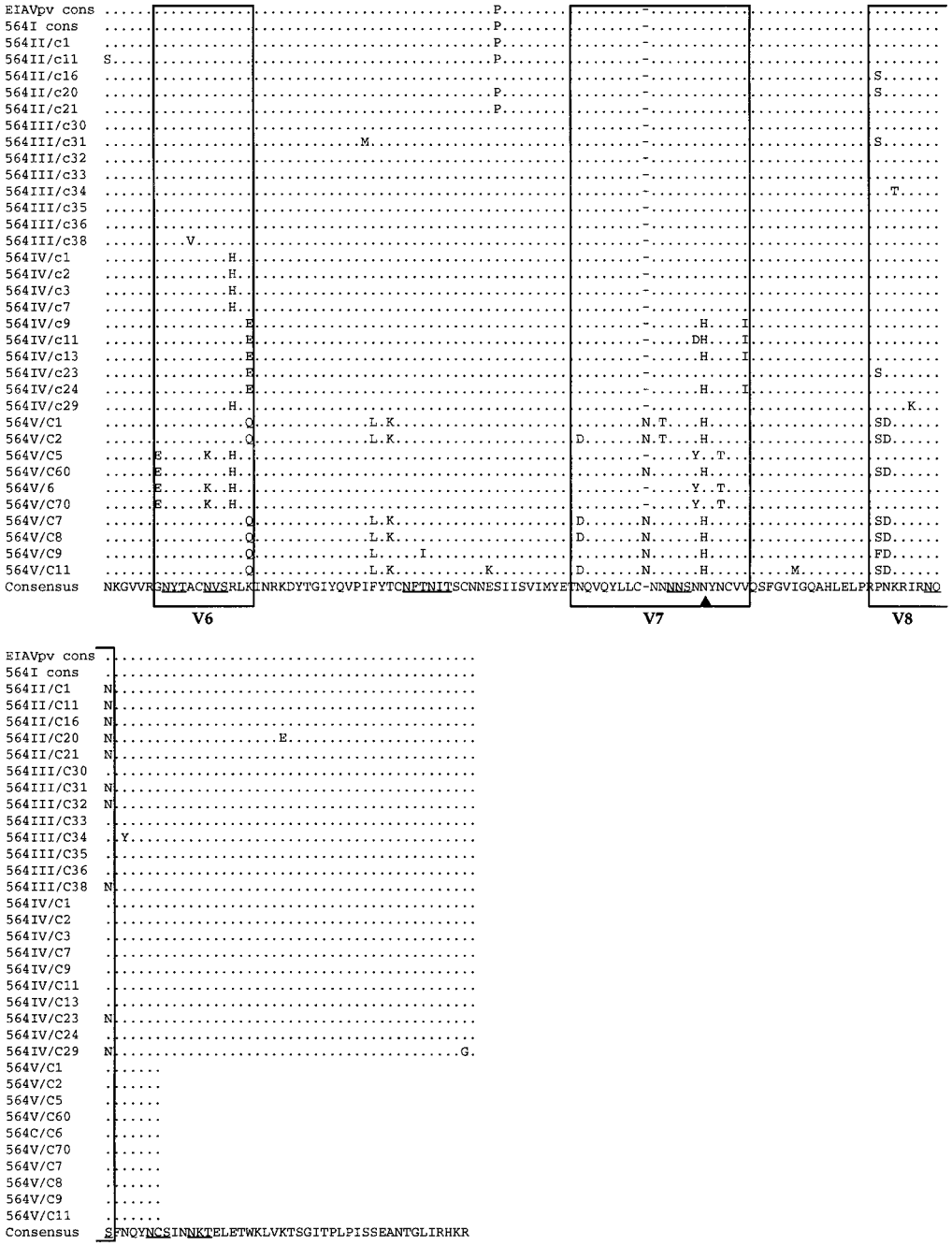


FIG. 5. Comparison of deduced amino acid sequences of EIAV gp90. The region of *env* gene (nucleotides 5322 to 6684 of pEIAV19-2) coding for the surface glycoprotein gp90 was sequenced from $EIAV_{PV}$ stock and plasma viral RNA obtained during febrile episodes I to V of $EIAV_{PV}$-infected pony 564. The deduced amino acid sequences were aligned by using the Genetic Computer Group sequence analysis package (9) and compared to the consensus (cons) sequence. Only the amino acids that differ from the amino acid consensus sequence are indicated. Since very few mutations were seen in the $EIAV_{PV}$ stock or during the first febrile episode (564I), only the amino acid consensus sequences were considered for these two samples. For febrile episodes II to V, amino acid sequences of all the individual clones are reported on the alignment. A dot indicates a amino acid identical to the consensus sequence; a dash indicates a deletion. The potential N-glycosylation sites are underlined on the consensus sequence. The created N-glycosylation sites are indicated (▲). Based on the amino acid sequence alignment of the gp90 sequences, eight gp90 variables regions (V1 to V8) were defined as stretches of amino acids with at least 30% divergence from the consensus sequence of the 43 clones. Only the mutations present in at least 2 of the 43 analyzed clones were considered to delineate the variable regions. The amino acid sequence described as the PND and located between two cysteine residues (C_{180} and C_{213} in the pEIAV19-2 sequence) is indicated by a thick black underline. The defined neutralizing epitopes E_{NT}, D_{NT}, and C_{NT} (3) are indicated by shaded boxes.

productive infection of fresh FEK cells cultures (data not shown).

Neutralizing properties of the *env*-deleted chimeric virus. Having established that the observed envelope deletions did not affect virus replication, we next sought to examine the effects of this particular envelope deletion on the serum neutralization properties of this virus envelope variant. Thus, we analyzed the neutralizing properties of the virus produced

aa:	348 EPI	568 SKM	580 TIL	726 EAF	754 GSG
EIAVpv	P (5/5)	K(3/4) R(1/4)	I(4/4)	A(4/4)	S(4/4)
564.I	P(10/10)	K(10/10)	I(10/10)	A(10/10)	S(10/10)
564.II	P(4/5) S(1/5)	K(5/5)	I(5/5)	A(5/5)	S(5/5)
564.III	S(8/8)	R(7/8) R(1/8)	I(3/8) T(3/8) V(2/8)	A(2/8) T(6/8)	S(5/8) A(3/8)
564.IV	S(10/10)	R(9/10) K(1/10)	V(10/10)	T(10/10)	A(10/10)
564.V	S(10/10)	ns	ns	ns	ns

<--- gp90 --><---------- gp45---------->

FIG. 6. Identification of hypervariable amino acid residues in gp90 and gp45. The positions of the hypervariable residues refer to pEIAV19-2 Env and are numbered from the ATG initiation codon. To facilitate their localization, the hypervariable amino acids are underlined and placed in the context of the flanking amino acid residues. ns, not sequenced.

from the 19.2/564IV.3 clone, using serum samples taken from pony 564 at 42, 84, 119, 182, 238, 301, and 364 days postinoculation. The results of these serum neutralization assays indicated that none of the serum samples were able to neutralize the virus (data not shown), although of number of these same serum samples displayed high levels of neutralization activity against the infecting $EIAV_{PV}$ strain of virus (Fig. 2B). These studies suggest that the PND deletion provides an escape from the existing serum neutralizing antibodies in pony 564 and, moreover, indicate that the host pony is unable to produce neutralizing antibodies to the deleted envelope, even 284 days after the appearance of this variant during the third febrile episode. These results indicate that serum neutralizing antibodies, at least as measured in vitro, were not responsible for bringing the viremia associated with febrile episodes III and IV under control and that other immune responses (cytotoxic T-lymphocyte [CTL] response, antibody-dependent cellular cytotoxicity, etc.) mediated the control of virus replication and disease. This lack of a correlation between serum neutralization activity and control of the episodic viremia and disease cycles was also observed during the first two febrile episodes in pony 564, in which $EIAV_{PV}$ replication was brought under control in the absence of detectable neutralizing antibodies against that virus strain, as reported previously (reference 12 and Fig. 2B).

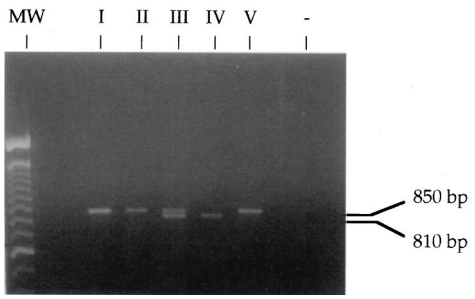


FIG. 7. gp90 length polymorphism demonstrated by RT-PCR amplification of plasma RNA. cDNAs prepared from plasma RNA taken during febrile episodes I to V were amplified by PCR using primers Var1 (sense) and Var2 (antisense) located in the gp90-encoding sequence of *env*. A large (about 850-bp) fragment and a small (about 810-bp) fragment were resolved on a ethidium bromide-stained agarose gel. MW, 100-bp DNA ladder; −, PCR negative control.

DISCUSSION

The well-defined clinical episodes observed during experimental EIAV infection provide a unique system with which to study the association between the genetic and antigenic variations and the progression of the disease in a lentiviral infection. Using our experimental $EIAV_{PV}$-infected pony system, we performed an extensive analysis of the genetic evolution of three functionally distinct regions of the EIAV genome: the *env* gene coding for the structural glycoproteins gp90 and gp45 that are predominant targets of host immune responses, the noncoding LTR region which regulates the replication of the virus, and the third open reading frame encoding the regulatory protein Rev.

After experimental infection of pony 564 with $EIAV_{PV}$, the viral populations present in the plasma at the time of each febrile episode were analyzed and compared to the inoculated strain. As previously described (22), the sequence diversity within the $EIAV_{PV}$ strain used for the inoculation was low likely due to the fact that $EIAV_{PV}$ is a biological clone (38). During the course of infection, however, nucleotide and amino acid mutations accumulated at about the same rate in gp90 and *rev* and to a lesser extent in gp45 and the LTR. While the variation observed in gp90 may be attributed to immune selection, the selective force for the EIAV Rev protein remains to be determined.

We observed in this study and in a previous study (22) that the U3 region of the LTR sequences (Fig. 3A) of $EIAV_{PV}$ and of the plasma-derived RNA lack the second CAAT motif and the third *ets* motif. Our results are in contrast with previous analyses suggesting that a second CAAT box is important for viral pathogenesis (5) and that a third *ets* motif is critical for high-level Tat-transactivated transcription in macrophages in vitro (23). Despite the absence of these motifs, $EIAV_{PV}$ is clearly pathogenic and able to replicate in macrophages, since these cells are the major target of EIAV infection in vivo. The previously described HVR of LTR among cell-adapted EIAV strains (5) is perfectly conserved in our 43 clones. These results support the idea that the previously described hypervariability may have occurred in vitro (23).

One of the more surprising results of this analysis is the in vivo generation of large deletions (up to 15 aa) in the region defined as the PND of EIAV (3). Variation in size of the surface glycoprotein between different isolates has been described for other lentiviruses such as HIV-1 (2, 43), BIV (46, 47), FIV (35), or small ruminant lentiviruses (21). No simple mechanisms, such as those described for BIV (47), seem to explain the generation of the 14- to 15-aa deletion observed during the third and fourth febrile episodes or the generation of a new complete PND during the fifth episode. With the low fidelity of the reverse transcriptase due to its absence of proofreading activity, recombination between copackaged RNA molecules has been proposed as one of the potential mechanisms to generate mutations in lentiviral genomes. This phenomenon has been found in vitro (15) and in vivo for HIV-1 (39, 49) and FIV (19). Deletions may occur during a recombination event or a strand jump during the reverse transcription of the RNA.

The observed deletions in the V3 region did not alter the competence for in vitro replication, as shown by the analysis of a *env* chimeric clone with a deleted PND, and did not alter the fitness of the virus in vivo, since the virus present during the third episode became the major population during the fourth febrile episode. This region of gp90 is probably not involved in the interaction of EIAV envelope with a specific, still unknown receptor (s) on the cell surface.

	V1 (aa 26-42)	V2 (aa 143-146)	V3 (aa 186-201)	V4 (aa 234-237)
Cons.	KCEENTMFQPYCYNND	SMHM	NSSDSSNPVRVEDVMN	DTDT
EIAVPV	V1a(5/5)	V2a(5/5)	V3a(3/5) ..... V3b(1/5) .....S..... V3c(1/5) .....K	V4a(5/5) ....
564I	V1a(10/10)	V2a(10/10)	V3a(8/10) ..... V3b(1/10) .....S..... V3c(1/10) .....K	V4a(8/10) .... V4b(1/10) ...A V4c(1/10) ...I
564II	V1a (1/5) ..... V1b (3/5) ....I..... V1c (1/5) .....I.....	V2a(2/5) .... V2b(1/5) ...I V2c(1/5) .N.. V2d(1/5) ..I.	V3d(1/5) .....L.....T. V3e(3/5) .....S.....I.. V3f(1/5) .....T.....I.	V4a(5/5) ....
564III	V1a (4/8) ..... V1d (1/8) ....I.....G V1e (1/8) ....I.....K. V1f (1/8) .....I.....G	V2b(4/8) ...I V2d(2/8) ..I. V2e(1/8) .NI. V2f(1/8) ...T	V3g(3/8) ----------.. V3h(1/8) ...G...S.....I. V3i(1/8) ----------. V3j(2/8) .....I. V3k(1/8) .....S.....I.	V4a(4/8) .... V4d(1/8) ..V. V4e(2/8) ..N. V4f(1/8) .N..
564IV	V1g(10/10) R.....	V2b(9/10) ...I V2f(1/10) ...T	V3g(9/10) ----------.. V3l(1/10) ----------...	V4f(10/10) .N..
564V	ns	V2g(7/10) ...I V2g(3/10) T..I	V3m(6/10) T..GP..S....YET. V3n(1/10) D..G---S.S...EI. V3o(2/10) T..G..IS..G..EI.	V4g(3/10) VN.. V4h(3/10) VNG. V4i(2/10) GN.. V4j(2/10) AN..

	V5 (aa 275-284)	V6 (aa 307-318)	V7 (aa 366-377)	V8 (aa 393-401)
Cons.	VQEKGIANTS	GNYTACNVSRLK	-NNNNSNNYNCVV	PNKRIRNQS
EIAVPV	V5a(5/5)	V6a(4/5) ..... V6b(1/5) .....R	V7a(5/5) -.....	V8a(4/5) ..... V8b(1/5) .....N
564I	V5a(9/10) ..... V5b(1/10) .R.....	V6a(10/10)	V7a(10/10) -.....	V8a(9/10) ..... V8b(1/10) .....N
564II	V5a(5/5)	V6a(5/5)	V7a(5/5) -.....	V8b(3/5) .....N V8c(2/5) S.....N
564III	V5a(8/8)	V6a(7/8) ..... V6c(1/8) ....V.....	V7a(5/8) -.....	V8a(5/8) ..... V8b(2/8) .....N V8c(1/8) S.....N
564IV	V5c(6/10) A..... V5d(4/10) ...G.....	V6d(5/10) .....H.. V6e(5/10) .....E	V7a(6/10) -..... V7b(3/10) -.....H....I V7c(1/10) -.....DH....I	V8a(8/10) ..... V8c(1/10) S.....N V8d(1/10) ....K...N
564V	V5e(1/10) ....R..... V5f(1/10) ....R..S.A V5g(1/10) ....ES.... V5h(3/10) .....S.... V5i(3/10) .....S.A V5J(1/10) .....S..	V6f(6/10) .....Q V6g(3/10) E.....K..H.. V6h(1/10) E.....H..	V7d(2/10) N.T....H..... V7e(3/10) -.....Y..T... V7f(5/10) N.....H.....	V8a(3/10) ..... V8e(6/10) SD..... V8f(1/10) FD.....

FIG. 8. Identification and characterization of the variable regions in gp90. Based on the amino acid sequence alignment of the gp90 sequences shown on Figure 7, eight variables regions (V1 to V8) were defined in gp90. The first and last amino acid residues of V1 to V8 refer to $EIAV_{PV}$ gp90 consensus (Cons.) sequence. The variable amino acid residues within the variable regions are underlined. For each variable region, the different sequences found in $EIAV_{PV}$ stock or in plasma of pony 564 are indicated (for example, V1a to V1g for V1). For each episode, the number of clones with a given genotype over the total number of sequenced clones is indicated in parentheses. ns, not sequenced.

The gp90 surface protein is one of the most rapidly evolving components of EIAV, as is the case with surface glycoproteins of other lentiviruses, such as primate lentiviruses (13, 27), FIV (1, 11, 28, 44), BIV (46), and small ruminant lentiviruses (17, 21). Based on a limited data base of envelope sequences, we initially divided gp90 into a C_N domain, a large variable domain, an HVR fragment within the variable domain, and a C_C domain (30, 31). Using the deduced amino acid sequences generated in this study, we found that the envelope mutations are not randomly distributed, and we delineated eight variable regions (V1 to V8), defined as amino acid sequences with more than 30% divergence from the consensus sequence. Our variable regions V1, V7, and V8 are in the previously reported constant regions, while V2 through V6 match previously described immunodominant regions (3). Recently, Zheng et al. described a similar gp90 variable region in virus isolates recovered from horses experimentally infected with a nonadapted virulent strain of EIAV, V70 (50, 51). To date, three principal neutralizing epitopes have been observed for EIAV gp90: E_{NT}, D_{NT}, and C_{NT} (3). The V3 region of gp90 is part of the PND region and encompasses the entire E_{NT} epitope. The C_{NT} epitope is partially contained in the V5 region. By its structure (cysteine loop), its function (PND), and its location in the SU protein, the EIAV V3 resembles HIV-1 V3, also shown to be highly variable. The analysis of the viral quasispecies present in each of the variable regions clearly shows that the initial $EIAV_{PV}$ quasispecies evolves into a new quasispecies by the time of the second febrile episode and that a new predominant quasispecies is associated with each of the recurring febrile episode (Fig. 5 and 8). Except in case of the V8b genotype in the V8 region, all viral populations present 337 days postinfection at the time of the fifth episode are different from the $EIAV_{PV}$ population used to infect the pony (Fig. 8).

Immune selection has been proposed for the rapid genera-

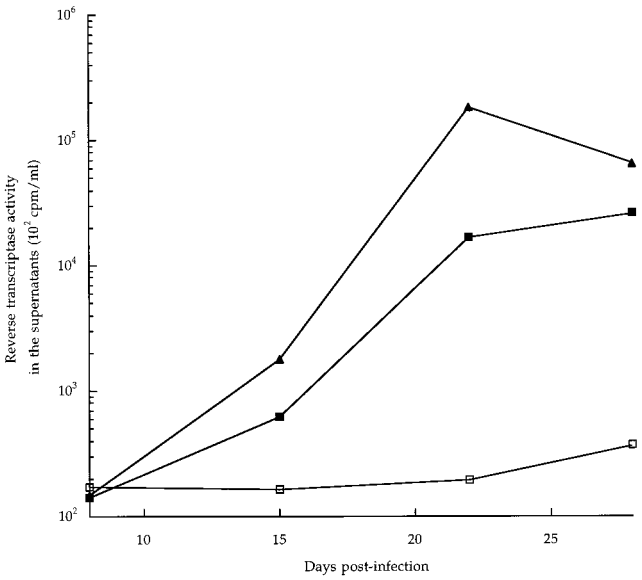


FIG. 9. Reverse transcriptase activity as a measure of viral replication in transfected FEK cells. Reverse transcriptase activity (*y* axis), as a measure of viral replication, was determined weekly (*x* axis) in FEK cells transfected with DNA of the *env* chimeric clone 19.2/564IV.3 (■), the wild-type pEIAV19-2 (▲), or Lipofectamine alone (□) as a negative control.

tion of antigenic variants of EIAV (38). However, the role and the importance of the immune system in the generation of envelope mutants remain unclear. In this study, gp90 mutants emerge in the plasma during the first (18 days postinfection) and the second (34 days postinfection) febrile episodes, in the absence of detectable neutralizing antibodies directed against the inoculated strain. As previously found in a horse infected with the Massachussets (MA1) strain of EIAV (4), our study of pony 564 suggests that neutralizing antibodies might not be involved in the generation of gp90 variants, at least in the early stage of the infection. But in contrast to Carpenter et al. (4), we show that the genomic mutations are not randomly distributed and that most of the defined variable regions are localized to immunodominant domains of gp90.

Pony 564 is characterized by an interesting pattern of the $EIAV_{PV}$-specific neutralizing antibodies in which detectable neutralizing antibodies appeared only after the third febrile episode, reached a maximum after the fourth febrile episode, and reappeared at the time of the late recrudescence of the disease, 337 days postinfection. The gp90 analysis showed that two of the three principal neutralizing epitopes (E_{NT} and C_{NT}) accumulated numerous amino acid mutations: during episodes II, III, IV, and V for E_{NT} and during episode IV for C_{NT}. The decline of $EIAV_{PV}$-specific neutralizing antibodies after the fourth febrile episode could be the result of the V3 deletion removing either the entire E_{NT} epitope of the PND or the point mutation observed in the C_{NT} epitope. The late recrudescence of the disease (episode V) is associated once again with a new viral strain containing a complete but distinct PND. The increase in neutralizing antibodies to the infecting $EIAV_{PV}$ observed during the fifth febrile episode suggests that envelope regions other than the described PND region are involved in determining envelope neutralizing properties. One of these regions might be C_{NT}, since this region accumulates during the fourth febrile episode mutations that could result in the decline of $EIAV_{PV}$-specific neutralizing antibodies and reverts to an $EIAV_{PV}$-like sequence during the fifth episode, at the time of the reemergence of $EIAV_{PV}$-specific neutralizing antibodies. A similar evolution of the CTL response occurs in this animal, in which Env-specific CTL emerge and decline during approximately the same time frame as the neutralizing antibodies (12). The previous CTL study in conjunction with the present study suggests that one or more of the gp90 or gp45 variable regions may be part of an immunodominant CTL epitope recognized by the pony immune system.

The present study demonstrates definitively for the first time that recurring episodes of EIAV are associated with the dynamic evolution and replication of distinct EIAV quasispecies that are present in the plasma during cycles of viremia and disease. Thus, these observations are consistent with the hypothesis that the recurring nature of chronic EIA is the result of the sequential evolution of EIAV antigenic variants that temporarily escape established immune surveillance (24, 25). The data further demonstrate that the ability of the immune system to control EIAV replication is the major determinant of disease in an infected animal. Thus, the current collection of sequential EIAV sequences provides an important panel of defined viral antigens to investigate in detail the critical relationship between envelope variation, host immune response, and levels of viral replication and disease during a persistent lentiviral infection.

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