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In Vivo Dynamics of Equine Infectious Anemia Viruses Emerging during Febrile Episodes: Insertions/Duplications at the Principal Neutralizing Domain

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Equine infectious anemia virus (EIAV) is a good model for studying mechanisms generating escaped retrovirus variants. We previously sequenced the entire gp90-encoding region of 22 cDNA clones obtained from five antigenically distinct isolates (F1V to F5V) recovered during febrile episodes in horse 493 experimentally infected with the Japanese virulent EIAV strain V70. The results showed that the mutations occurred in the principal neutralizing domain (PND) by insertions/duplications. In this study, we further characterized the PND of virus isolates sequentially recovered during 22 febrile episodes in seven horses newly infected with V70 or one of the V70-derived variants. Sequencing of 70 cDNA clones derived from the 22 episodes confirmed the generation of various new viral quasispecies with insertions/duplications in the PND. Although the insertion/duplication sequences in a total of 92 cDNA clones were extensively heterogeneous, we hypothesized that all the insertions/duplications occurred during reverse transcription from viral genomic RNA to minus strand DNA. The insertion/duplication regions were derived from a part of the PND sequence, which consisted of five small units. These small units, some with various substitutions and/or deletions, were also generated, especially in regions with insertions/duplications. Of particular note was that all these virus variants, except for two cDNA variants, were generated by essentially four different duplication pathways. Thus, these results extend the significance of insertions/duplications in the PND to the novel generation of EIAV in vivo during febrile episodes.

Human immunodeficiency virus type 1 (HIV-1) induces the slow progressive course of AIDS. Initial viremia after primary infection with HIV-1 is followed rapidly by viral clearance, concomitantly with a specific cell-mediated immune response (15, 23). Thereafter, the persistence of low levels of HIV-1 in peripheral blood is facilitated for years before the onset of the disease. The clinical stage is significantly associated with virus load (5, 18). A high absolute number of infected cells were detectable in lymphoid tissues throughout the early to late stages of infection (7, 8, 24). Further, the dynamics of HIV-1 replication in vivo has recently been revealed by quantifying serial changes in the viral genotype and phenotype with respect to drug resistance in plasma and peripheral blood mononuclear cells (30, 39). Thus, knowledge of the mechanisms of serial changes in the genotype and phenotype of lentiviruses is essential for understanding the pathogenicity of the disease.

Equine infectious anemia virus (EIAV) provides a unique system for the study of the mechanisms and roles of genomic variation in viral persistence and pathogenesis, because of its rapid antigenic variation compared to that of the other lentiviruses (16, 35). It has been demonstrated that serum antibodies from an EIAV-infected horse can neutralize virus isolates recovered from prior, but not subsequent, disease episodes, each of which is associated with a novel predominant antigenic variant (14, 19). Tryptic peptide and glycopeptide maps and oligonucleotide fingerprinting revealed alterations in the amino acid sequence and glycosylation pattern of the Env protein in these variants (21, 28, 29, 34). Rapid alterations of epitope within Env gp90 have also been documented by using monoclonal antibodies (10). Sequence analysis of the predominant viruses isolated from sequential febrile episodes in a single pony showed that much of the variation in the gp90 gene was clustered in a single area termed the variable region, especially in the restricted site, named the hypervariable region (HVR) (26, 27). In addition, Ball et al. (2) identified a principal neutralizing domain (PND) in the gp90 gene variable region with neutralizing monoclonal antibodies. Thus, the accumulated evidence may indicate sequential generation of variants by mutations in the PND and HVR during disease episodes.

Recently, we compared the sequences of the entire gp90 gene among various virulent and avirulent strains of EIAV and defined six variable domains (V1 to V6): V3 and V5 correspond to PND and HVR, respectively (40). Lichtenstein et al. (16) revealed that the EIAV genomic quasispecies associated with the first clinical episode in two ponies infected with pathogenic strain $EIAV_{pv}$ are mainly due to substitutions in the PND and HVR. Our sequencing study on the full-length gp90 gene of five antigenically distinct isolates recovered during sequential febrile episodes in horse 493 experimentally infected with the Japanese virulent strain V70 also confirmed drastic mutations in the PND and substitutions in the HVR (40). The mutation mechanisms in the PND were insertions/duplications of three small units located just before the inser-

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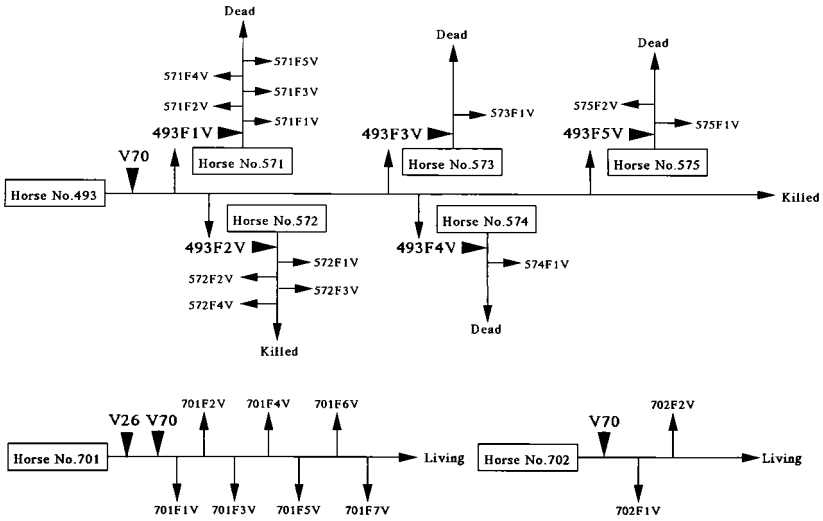

FIG. 1. Schedules for EIAV infections. Horses 571, 572, 573, 574, and 575 were infected with 493F1V, 493F2V, 493F3V, 493F4V, and 493F5V, respectively. Thirteen virus isolates (571F1V, 571F2V, 571F3V, 571F4V, 571F5V, 572F1V, 572F2V, 572F3V, 572F4V, 573F1V, 574F1V, 575F1V, and 575F2V) were obtained from the sera of these horses at sequential febrile episodes. These virus isolates were propagated in primary horse macrophage cultures. The cellular DNAs were subjected to PCR. Horse 701 was immunized with avirulent EIAV strain V26 and then challenged with cell-associated V70. Horse 702 was infected with cell-free V70. Nine virus particle fractions prepared from the plasma samples from each febrile episode in these two horses (701F1V, 701F2V, 701F3V, 701F4V, 701F5V, 701F6V, 701F7V, 702F1V, and 702F2V) were used for RNA extraction. The RNA samples were subjected to RT-PCR.

tion region (40). These results strongly suggest that the mutations in the PND and HVR could be quite important for the emergence of sequential variants and thus for disease progression.

In this study, we further characterized the gp90 gene variable regions of EIAVs from 22 febrile episodes in seven horses newly infected with V70 or one of the V70-derived variants in order to extend the in vivo mutation mechanism for the PND and HVR. Sequencing of a total of 70 cDNA clones revealed that the PND sequences contained insertions/duplications, similar to the 22 cDNA clones from horse 493 (40). Such insertions/duplications seemed to occur during reverse transcription to the minus strand during DNA synthesis, and therefore the insertion/duplication sequences could be derived from a part of the PND sequence (consisting of five small units, SU1 to SU5) of another template viral RNA. Consequently, the PND sequences in a total of 97 cDNA clones, including five cDNA clones from V70-infected macrophages, were classified into four (I to IV) types, which seemed to be produced by different duplication pathways, although the small units used for the insertions/duplications had substitutions and/or deletions, compared with the original small units.

MATERIALS AND METHODS

Viruses. The virulent strain of Japanese EIAV (named V70) (12) and variants derived in vivo from V70-infected horse 493 (named 493F1V, 493F2V, 493F3V, 493F4V, and 493F5V) (14) have been described previously. The attenuated strain of EIAV, V26, which was obtained after 50 passages of V70 in primary horse macrophage cultures, has been described previously (12).

Virus infection to and isolation from horses. Horses 571 to 575 were newly infected with cell-free 493F1V to 493F5V, respectively (see Fig. 1). Thirteen viruses (named 571F1V to 571F5V, 572F1V to 572F4V, 573F1V, 574F1V, 575F1V, and 575F2V) were isolated by culture of primary macrophages with EIAV in the sera during each of the febrile episodes from those horses, as described previously (40). On the other hand, horse 702 was infected with cell-free V70 (see Fig. 1). Furthermore, horse 701 was initially inoculated with avirulent cell-free V26 and then subsequently challenged with cell-associated virulent V70 in primary macrophages derived from this horse (see Fig. 1). The viruses, tentatively named 701F1V to 701F7V, 702F1V, and 702F2V, in the plasma samples from the nine febrile episodes seen in these two horses were also characterized by nucleotide sequencing.

PCR. Primary horse macrophage cultures were inoculated with virus isolates from horses 571 to 575. The cellular DNAs were subjected to PCR for amplification of the region covering the PND and HVR with the primer pair EPND-D2 (5′-GTCAAGAGTATCAATGTCAA-3′) from nucleotides 5801 to 5820 and EHPR-A1 (5′-GGAAGTTATGTTAGTGAAAT-3′) from nucleotides 6306 to 6287, as described previously (40). On the other hand, virion RNA was subjected to reverse transcriptase (RT)-PCR. The viruses in the plasma from horses 701 and 702 were pelleted by ultracentrifugation at 70,000 rpm with a TLA-100.3 rotor (Beckman) for 30 min. Total RNA fractions were extracted with an RNA extraction kit (Isogen; Nippon Gene Co., Tokyo, Japan). Total RNA (1 μg) was reverse-transcribed with RNase H-negative reverse transcriptase (Superscript II; Gibco BRL), as specified by the manufacture. The first-strand synthesis was initiated with an EIAV-specific primer, EHPR-A1 (0.1 μM). Five microliters containing synthesized cDNA was similarly subjected to PCR in a 100-μl reaction mixture (Perkin-Elmer), as described above.

Cloning of PCR products and sequencing. The amplified PCR and RT-PCR fragments were directly cloned into the pCR vector (Invitrogen Corporation, San Diego, Calif.). The DNA clones were screened by colony PCR with M13 forward and reverse primers. Briefly, the *Escherichia coli* colonies were dissolved in 100 μl of distilled water containing 0.1% Tween 20 and boiled for 15 min. Ten microliters of the supernatant was used as a template in 100 μl of PCR mixture (Perkin Elmer), and the incubation profile was 1 cycle of 4 min at 94°C and 30 cycles each of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C. The positive-colony-PCR fragments were purified with a Wizard PCR Preps DNA purification system (Promega) and used as sequencing templates. Sequencing was performed with a Dye Primer Cycle Sequencing Ready Reaction kit (Applied Biosystems) with either −21 M13 or M13 reverse. Sequences were resolved with an ABI 373A DNA sequencer (Applied Biosystems). The sequences were analyzed with GENETYX-MAC DNA sequence analysis software (Software Development Co., Tokyo, Japan).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the following accession numbers: D87640 to D87656 and D87719 to D87733 for cDNA clones from horse 493 as described previously (40); D89380 to D89395 for horse 571; D89396 to D89409 for horse 572; D89410 to D89412 for horse 573; D89413 to D89415 for horse 574; D89416 to D89421 for horse 575; D89422 to D89429, D89480 to D89485, and AB000191 to AB000198 for horse 701; and D89486 to D89491 for horse 702.

RESULTS

EIAVs during sequential febrile episodes in Japanese virulent strain V70- or V70-derived-variant-infected horses. Five virus isolates (named 493F1V, 493F2V, 493F3V, 493F4V, and 493F5V) from periodic febrile episodes of V70-infected horse 493 were used to infect horses 571 to 575, respectively (Fig. 1).

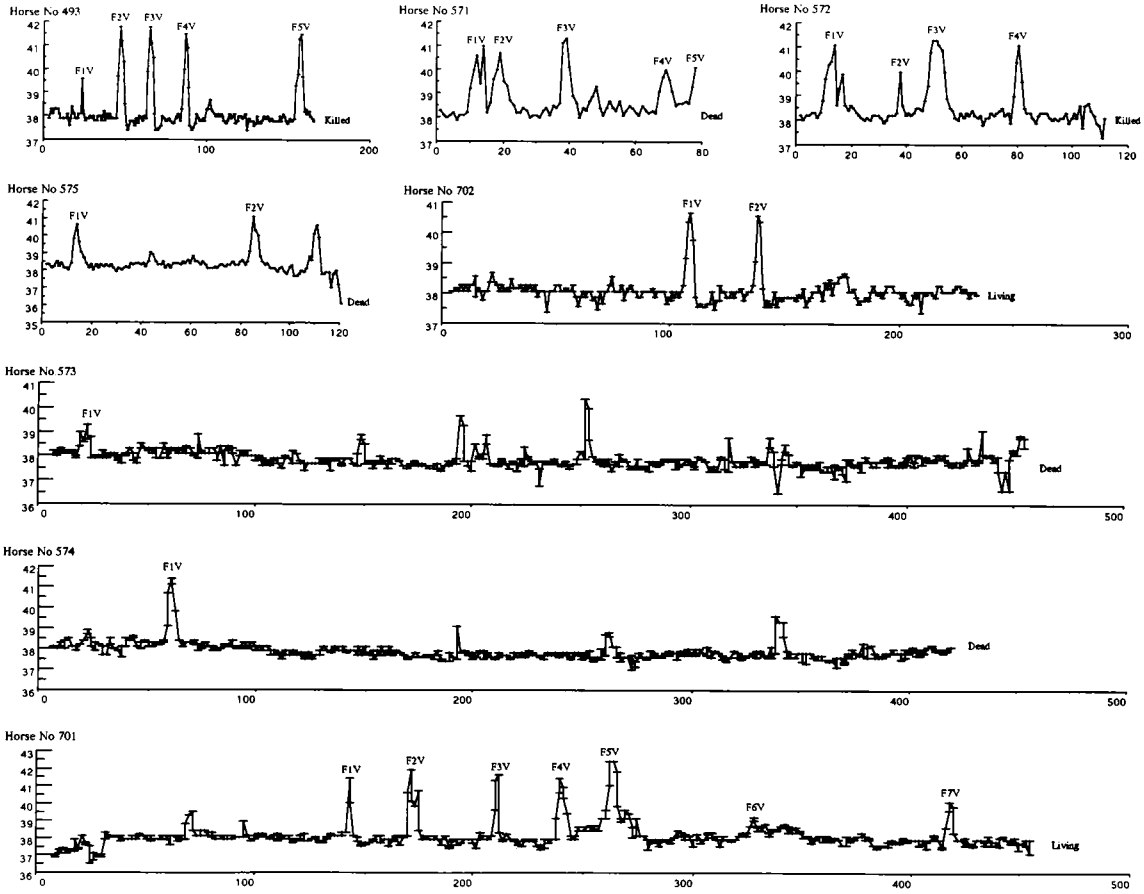

FIG. 2. Clinical history of the eight V70- and V70-derived-variant-infected horses. Horses 493, 571, 572, 573, 574, 575, 701, and 702, after infection as described in the legend to Fig. 1, developed clinical diseases characterized by periodic febrile episodes. Longitudinal axis, body temperature (°C); latitudinal axis, number of days postinfection. Viruses or plasma samples were isolated at each peak of body temperature (febrile episode).

These horses also showed typical clinical signs with periodical relapses of fever (Fig. 2). A total of 13 viruses were isolated from the sera of these horses at each febrile episode by inoculation into primary horse macrophage cultures with these sera: 571F1V to 571F5V from horse 571 infected with 493F1V; 572F1V to 572F4V from horse 572 infected with 493F2V; 573F1V from horse 573 infected with 493F3V; 574F1V from horse 574 infected with 493F4V; and 575F1V to 575F2V from horse 575 infected with 493F5V. In order to identify the sequence differences in the PND and HVR, the variable region including these two regions of the viruses in infected cells was amplified by PCR. The amplified products were cloned into a pCR vector, then sequenced.

Generally, the predominant population of replication-competent viruses is reflected by the viruses isolated in cultures, as described above. However, for HIV-1 there is also evidence that genetic data obtained with virus isolates in vitro have not always correlated with that obtained in vivo (17). Therefore, it is likely that the total population of currently replicating viral species in vivo at each febrile episode would be reflected by the way the viruses are directly captured in the plasma by the RT-PCR technique (16). To examine this possibility, we infected another horse, 702, with cell-free V70 to analyze the PND and HVR sequences of the EIAV in the plasma by RT-PCR (Fig. 1). This horse also showed clinical episodes (Fig. 2). The RNAs (named 702F1V and 702F2V) extracted from the plasma during episodes were subjected to RT-PCR and then cloned into a pCR vector. In addition, horse 701 was first inoculated with attenuated cell-free V26, followed by a challenge with cell-associated virulent V70 (Fig. 1). Horse 701, inoculated with V26, showed a mild febrile episode before being challenged with V70 on day 65 (Fig. 2). However, there was no amplification of EIAV in the plasma obtained from this febrile episode (data not shown), suggesting that this febrile episode was not due to V26 replication. In fact, we can identify that this febrile episode was due to laminitis. When the horse was challenged with cell-associated V70, a total of seven typical clinical episodes were induced (viruses 701F1V to 701F7V) (Fig. 2). The EIAVs in the plasma during these episodes were amplified by RT-PCR and then cloned into a pCR vector.

Sequences of new EIAV quasispecies at variable regions including the PND and HVR. The region amplified in this study contained three variable domains (V3 in the PND, V4, and V5 in the HVR) and the codons for two hypervariable amino acids (L^{219} and S^{235}), which were identified previously by comparison of V70-derived variants with the reported *env* gp90 sequences (40). Figure 3 shows the deduced amino acid sequences for a total of 70 cDNA clones derived from EIAVs at 22 febrile episodes shown by seven horses. The previously reported sequences for 22 cDNA clones derived from five febrile episodes from horse 493 as well as 5 cDNA clones from V70-infected macrophages are also shown in this figure. The PND in particular showed drastic mutations, including insertions/duplications and substitutions. 572F2V,

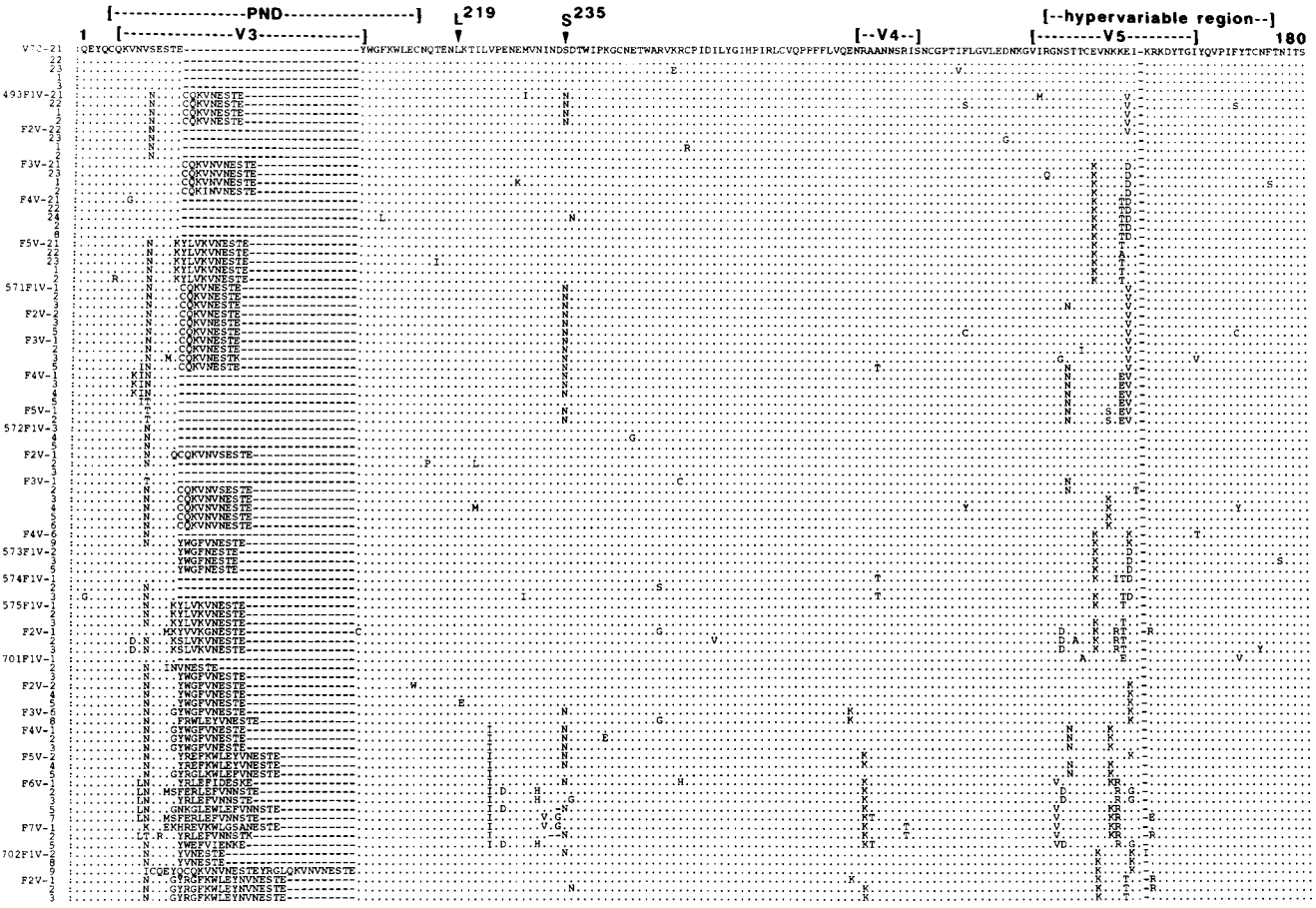

FIG. 3. Deduced amino acid sequences of the EIAV gp90 gene variable region from the PND to the HVR in cDNAs derived from EIAVs during clinical episodes in eight horses. Seventy cDNA clones were obtained from EIAVs during 22 clinical episodes in seven newly infected horses. In addition, 22 reported sequences from five febrile episodes in horse 493 and 5 from V70-infected macrophages (40) are also shown. The clone V70-21 amino acid sequence is shown. Points indicate identity with the V70-21 sequence and dashes indicate gaps. The PND, HVR, and V3 to V5 domains are indicated. Two hypervariable amino acid residues, L^{219} and S^{235}, are marked. cDNA clones are indicated on the left.

572F3V, 572F4V, 701F1V, 701F3V, 701F6V, 701F7V, and 702F1V were mixtures of two different-sized PND sequences. In contrast, the HVR was mildly mutated, mainly by substitution, except for one clone, 702F1V-2, which contained a mutation resulting in an amino acid insertion. Slightly fewer substitutions were observed in the V4 domain. The hypervariable amino acid L^{219} remained for all the clones, whereas S^{235} was changed to N^{235} for most of the clones. These results are essentially in accordance with the mutations seen in virus isolates (F1V to F5V) from V70-infected horse 493 (40). In addition, a high number of mutations by substitution, including deletions of one or two amino acids for two clones, was observed at a region between L^{219} and S^{235} for the cDNA clones derived from 701F4V, 701F5V, 701F6V, and 701F7V, especially for the latter two clones.

Sequence analysis of the PND. In order to clarify a possible regular mechanism for PND mutation, we characterized PND sequences by using small nucleotide sequence units. The PND sequence in V70 was divided into six small units: TGTCAA AAA (named SU1), GTTAAT (SU2), GTTAGT (a derivative of SU2), GAGAGTACGGAA (SU3), TATTGGGGATTT (SU4), and AAATGGCTAGAATGT (SU5). Similarly, all the PND sequences in 92 cDNA clones were also divided into similar small units. According to this definition, a total of four types (I to IV) of PND composition, which differed in size, were identified among these clones, except for 701F1V-2 and 702F1V-9 (Fig. 4). The PND compositions were SU1-SU2-SU2-SU3-SU4-SU5 for type I, SU1-SU2-SU2-SU3-SU1-(SU2)-SU2-SU3-SU4-SU5 for type II, SU1-SU2-SU2-SU3-SU4-(SU2)-SU2-SU3-SU4-SU5 for type III, and SU1-SU2-SU2-SU3-SU4-SU5-(SU2)-SU2-SU3-SU4-SU5 for type IV. (Parentheses for SU2 indicate SU2s present in the corresponding virus type.) The PND composition type I, which includes the PND sequence of the original V70, was the shortest one, containing only one duplication of SU2, whereas types II to IV contained several duplications of SU1 to SU5.

The mutant forms of SU1 to SU5, with several substitutions and/or deletions, were also found in these PND sequences. For example, there were six isoforms of SU1 (SU1A to SU1F), 16 of SU2 (SU2A to SU2P), 15 of SU3 (SU3A to SU3O), 17 of SU4 (SU4A to SU4Q), and 10 of SU5 (SU5A to SU5J), as shown in Fig. 5. All mutant forms of SU1 were made by base substitution, whereas those of SU2 to SU5 were made by base deletion as well as substitution. There were seven deletion forms of SU4, but only one deletion form of SU2, SU3, and SU5.

Proposed model for the mechanism of insertions/duplications in the PND. It was previously hypothesized that misincorporation-related HIV-1 RT pausing during minus strand DNA synthesis allows the accompanying RNase H activity to

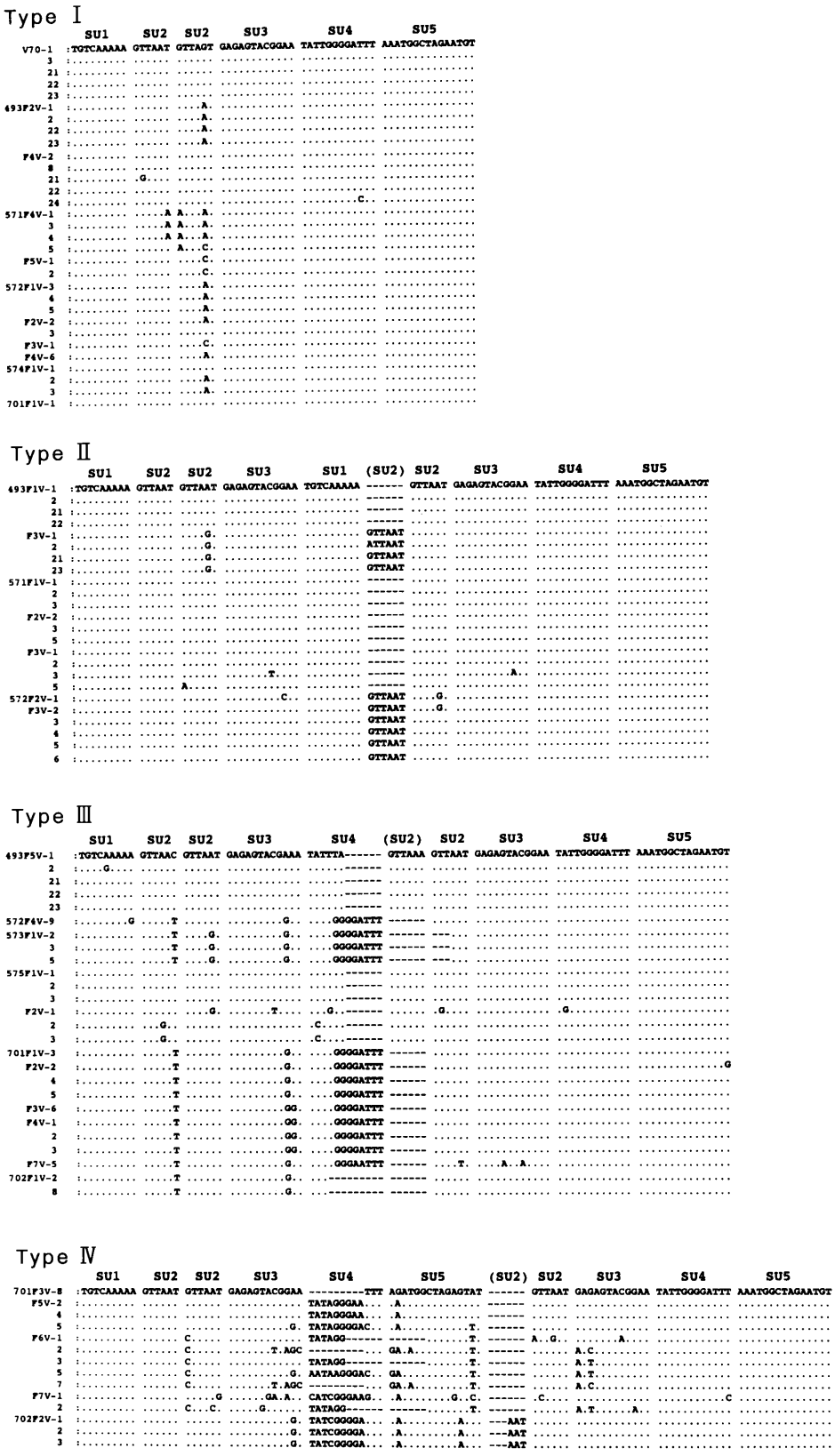

FIG. 4. Four types (I to IV) of PND nucleotide sequences derived from the EIAVs during clinical episodes in eight horses are shown along with the different compositions of their small unit (SU1 to SU5) nucleotide sequences. The nucleotide sequences of the cDNA clones whose deduced amino acid sequences are shown in Fig. 3 are shown. Points indicate identity with the top sequences and dashes indicate gaps.

SU1	SU2	SU3	SU4	SU5
SU1A: TGTCAAAAA	SU2A: GTTAAT	SU3A: GAGAGTACGGAA	SU4A: TATTGGGGATTT	SU5A: AAATGGCTAGAATGT
SU1B: TGTCAAAAG	SU2B: GTTAAA	SU3B: GAGAGTACGGGA	SU4B: TATTGGGGATTC	SU5B: AAATGGCTAGAATGG
SU1C: TGTCAAGAG	SU2C: GTTAAC	SU3C: GAGAGTACGAAA	SU4C: TATTGGGGACTT	SU5C: AAATGGCTAGAATAT
SU1D: TGTCGAAAA	SU2D: GTTAAG	SU3D: GAGAGTACGCAA	SU4D: TATTGGGAATTT	SU5D: AAATGGCTAGAGTAT
SU1E: TATCGGGGA	SU2E: GTTAGT	SU3E: GAGAGTATGGAA	SU4E: TATCGGGGATTT	SU5E: AAATGGCTAGGGTCT
SU1F: TTGCAAAAA	SU2F: GTTACT	SU3F: GAGAGTAAGGAA	SU4F: TATAGGGGACTT	SU5F: AAATGGCTAGAGTTT
	SU2G: GTTATT	SU3G: GAGAGTATGAAA	SU4G: TATAGGGAATTT	SU5G: AGATGGCTAGAGTAT
	SU2H: GTTGAC	SU3H: GAGAGTATGAGC	SU4H: TGTTGGGGATTT	SU5H: GAAAGGCTAGAGTTT
	SU2I: GGTAAT	SU3I: GAGAGTGAGAAA	SU4I: CATCGGGAAGTT	SU5I: GAATGGCTAGAGTTT
	SU2J: GCTAAT	SU3J: GAGAGGACGGAA	SU4J: AATAAGGGACTT	SU5J: ------CTAGAGTTT
	SU2K: ATTAAT	SU3K: GAGAATAAGGAA	SU4K: TATAGG------	
	SU2L: CTTAAT	SU3L: AACAGTACGGAA	SU4L: TATTTA------	
	SU2M: ATTACT	SU3M: AATAGTACGGAA	SU4M: TATGTA------	
	SU2N: CTTACT	SU3N: AATAGTACGAAA	SU4N: TCTTTA------	
	SU2O: ATTGAT	SU3O: GAGAGT------	SU4O: TATCAA------	
	SU2P: ---AAT		SU4P: ---------TTT	
			SU4Q: TAT---------	

FIG. 5. Summarized nucleotide sequences of the small units that appeared in the PND of the EIAVs during clinical episodes in eight horses. According to the results shown in Fig. 3 and 4, there are 6 isoforms of SU1 (A to F), 16 of SU2 (A to P), 15 of SU3 (A to O), 17 of SU4 (A to Q), and 10 of SU5 (A to J). The base substitutions are marked by outlines, and dashes indicate gaps.

degrade the template viral RNA beneath the growing primer to a greater extent than during normal synthesis (22). This cleavage can then promote recombination by RT-mediated transfer to another viral RNA template. This mechanism might be useful as one of the possible explanations for the insertions/duplications which were seen in the variants which emerged in vivo at sequential clinical episodes. Therefore, we proposed a model for the mechanism of the insertions/duplications in the PND that is in accordance with the above hypothesis. As shown in Fig. 6, type I is generated by the V70-type pathway: tandemly reverse-transcribed products from SU5 to SU1. In contrast, we suggest that EIAV RT elongates the primer up to SU1 in type II, and up to the first or second SU2 in types III and IV, and then pauses, eventually leaves the template, and transfers to another viral template RNA at the sites, such as SU3 in type II, SU4 in type III, and SU5 in type IV, for subsequent elongation. Based on this model, the sequences transcribed after transfer to another RNA template were SU1-SU2-SU2-SU3 in type II, SU1-SU2-SU2-SU2-SU3-SU4 in type III, and SU1-SU2-SU2-SU3-SU4-SU5 in type IV. Consequently, the insertions/duplications occurred in the regions underlined. Type I sequences contained two units of SU2, while the SU2 transcribed before the transfer was often contained only once in types II, III, and IV. Various mutant forms of the small

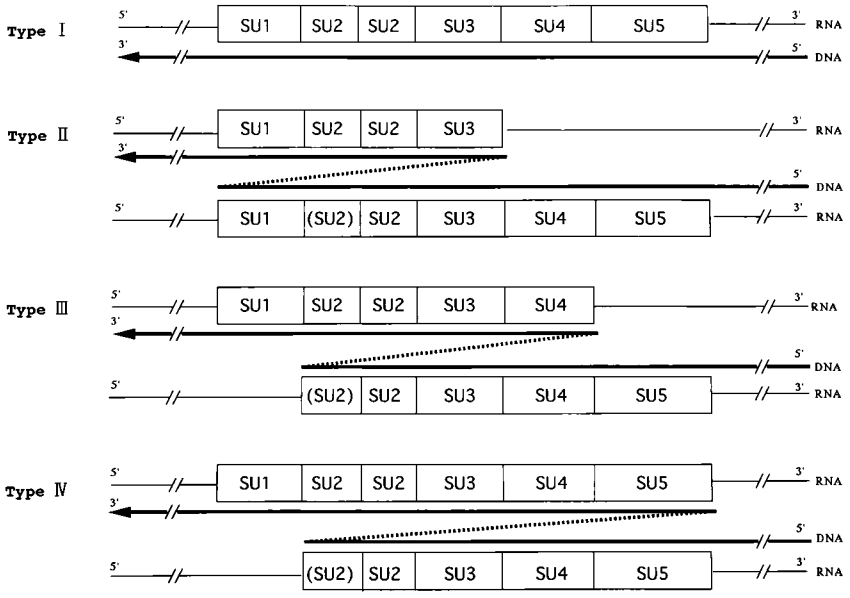

FIG. 6. A proposed model accounting for insertions/duplications in the PND in EIAV populations during clinical episodes in eight horses. The PND sequences in the EIAVs during clinical episodes in the eight horses infected were classified into four (I to IV) types, which were generated by different reverse transcription pathways. The mechanism for the insertions/duplications at the PND could be explained by RT-mediated transfer to another viral template RNA. Type I is generated by the V70-type pathway: tandemly reverse-transcribed product from SU5 to SU1. In contrast, EIAV RT elongates the primer up to SU1 in type II, up to the first or the second SU2 in types III and IV, and then transfers to another template RNA at SU3 in type II, SU4 in type III, and SU5 in type IV for subsequent elongation.

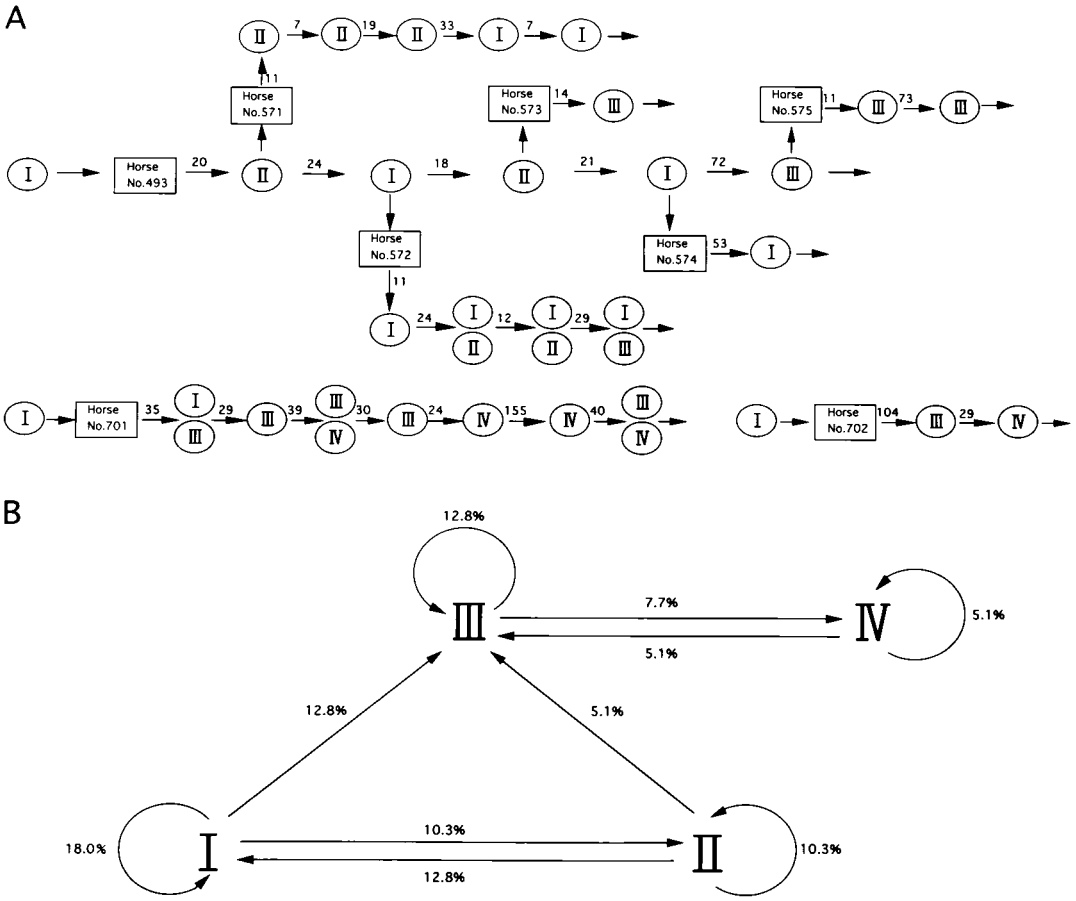

FIG. 7. Summarized results for various emerging variants during clinical episodes in V70- or V70-derived-variant-infected horses. (A) Eight horses (boxed) were infected with V70 or one of the V70-derived variants (arrowhead) and developed sequential clinical episodes. The different types of virus (circled) during clinical episodes (arrow) were subjected to cDNA cloning. The number of days between clinical episodes is shown beside the arrows. (B) The viral shifts observed between two sequential clinical episodes in eight horses are summarized. The percentages of individual shift, which were calculated according to the results shown in panel A, are also indicated.

units, compared with the original V70, were predominantly identified within insertions/duplications, and some were identified within the regions transcribed before transfer to another RNA template, especially in types III and IV (Figs. 4 and 5).

No unrestricted generation of EIAV variants in vivo. It is quite interesting to determine the restriction of the in vivo evolution of EIAV for the generation of subsequent variant viruses. The EIAV variants found during 27 febrile episodes in eight horses are summarized in Fig. 7. Comparison of sequential virus samples, with a total of 97 cDNA clones of the PND sequences, revealed that PND type I viruses were followed by the emergence of type II and III viruses which induced the next clinical episodes but not by type IV virus populations. Similarly, the type II viruses were followed by type I and III viruses but not by type IV virus populations. The type III viruses were followed by type IV viruses but not by type I or II virus populations. The type IV viruses could generate type III but not type I or II virus populations. All the virus types could induce the following episodes by virus populations of the same type. The average times between episodes were 97 days for type IV to IV, 54 days for type I to III, 36 days for type III to III, 35 days for type IV to III, 31 days for type III to IV, 24 days for types I to I and II to I, 22 days for type II to III, 19 days for type I to II, and 12 days for type II to II (Fig. 7A). The rates of virus type shift in the next episodes were 18.0% for type I to I; 12.8% for I to III, II to I, and III to III; 10.3% for I to II and II to II; 7.7% for III to IV; and 5.1% for II to III, IV to III, and IV to IV (Fig. 7B).

DISCUSSION

Since antigenic drift was revealed in the EIAVs that sequentially emerged in infected horses (14), the possible generation of new viral envelope mutants has been investigated to clarify the mechanism for the generation of escape variants (1, 10, 16, 19, 21, 27–29, 40). There have been several reports regarding mutations within *env* gp90 during febrile episodes in infected horses (1, 16, 27, 40). One of the conclusions is that the PND and HVR of *env* gp90 could be responsible for the antigenic drift, as variants with mutations in two such regions could escape from the host immune pressure and cause another clinical episode (16, 40).

Our previous *env* gp90-sequencing results obtained with 22 cDNA clones from variants associated with five febrile episodes in horse 493 infected with the Japanese EIAV virulent strain V70 revealed insertions/duplications in the PND and substitutions in the HVR (40). Therefore, we focused on the mechanisms of the mutations in the PND with a total of 70 cDNA clones obtained from viruses during 22 febrile episodes in seven newly infected horses (Fig. 1 and 2). Again,

sequencing of the PND and HVR revealed similar drastic mutations: insertions/duplications in the PND and substitutions in the HVR (Fig. 3). The sequences obtained by RT-PCR of EIAVs in plasma were almost similar to those obtained by PCR of isolated viruses. Thus, the predominant population of EIAVs at febrile episodes seemed to be reflected by the viruses with these sequences.

Characterization of the PND sequences (Fig. 4) revealed that this domain consisted of five small units (SU1 to SU5). All the PND sequences from the 97 cDNA clones (70 were newly prepared from 22 episodes in seven horses (Fig. 1), 22 from 5 episodes in horse 493, and 5 from V70-infected macrophages [40]), except those for 701F1V-2 and 702F1V-9, were classified essentially into four types (types I to IV) via four different transcription pathways: type I, SU1-SU2-SU2-SU3-SU4-SU5 (no insertion as V70); type II, SU1-SU2-SU2-SU3-SU1-(SU2)-SU2-SU3-SU4-SU5; type III, SU1-SU2-SU2-SU3-SU4-(SU2)-SU2-SU3-SU4-SU5; and type IV, SU1-SU2-SU2-SU3-SU4-SU5-(SU2)-SU2-SU3-SU4-SU5 (Fig. 6). The underlined portions seem to be the insertion/duplication regions. Further, many isoforms of the small units produced by base substitutions and base deletions were also identified, predominantly in the insertion/duplication regions, and some were identified in the regions transcribed before the transfer to another RNA template in types III and IV (Fig. 4); in total there were 6 isoforms of SU1, 16 of SU2, 15 of SU3, 17 of SU4, and 10 of SU5 (Fig. 5). Thus, all the PND sequences, except for two, were clearly classified into four types, although the PND sequences, even in the same type, were very heterogeneous. This heterogeneity is mainly due to the generation of isoforms of the small units.

RT is a multifunctional enzyme having RNA- and DNA-dependent DNA polymerase, RNase H, strand displacement, and strand transfer activities (9). The lack of an associated 3′-to-5′ exonuclease for proofreading and the propensity of RT to extend a mismatched primer on a DNA or an RNA template are thought to be the sources of variation in the retroviral genome (33). In fact, retroviruses mutate at high rates during the reverse transcription of the viral genomic RNA to proviral DNA and become variants with base pair substitutions, frameshifts, deletions, and deletions with insertions (6, 32, 37, 38). Recently, Palaniappan et al. (22) hypothesized that a misincorporation during synthesis of the minus strand DNA would cause HIV-1 RT to pause. This pause would lead to enhanced RNase H-directed cleavage of the template near the pause site. This cleavage should then promote RT-mediated transfer to another viral RNA template. In accordance with this hypothesis, we examined a possible explanation of the mutation mechanism for the generation of new EIAV quasispecies. In our case, the sequences at insertions/duplications consisted of small units with mutations (Fig. 4). In addition, the regions transcribed before the transfer to another RNA template in types III and IV also consisted of small units with mutations (Fig. 4). These results suggest that such transfer to different regions is the mechanism for generation of type II to type IV virus populations by subsequent elongation according to the sequence of another viral RNA template (Fig. 6). The mechanism of binding to specific regions which varied in types II to IV remains to be clarified. Alternatively, it might be possible that the insertions/duplications observed in this study might be derived from the error rate of RNA polymerase II during transcription from provirus DNA, as discussed previously (6). However, this is not likely in this case, because at least two copies of template would be necessary for insertions/duplications. Large deletions often occurred during retrovirus replication (11, 20). This kind of mutation may arise during reverse transcription (31) or plus strand DNA synthesis (25) and usually involves removal of nucleotides between small direct repeats (37). This may have also occurred in our system during the virus type change from the insertion/duplication form to the non-insertion/duplication form, i.e., from type II to I.

It would be interesting to clarify the limitations of the in vivo evolution of EIAVs. The viral shift at the next episode was found to be restricted by comparison of the PND sequences of 33 viruses from 27 episodes in eight V70- or V70-derived-variant-infected horses, i.e., there were no changes from type III to I and II or from IV to I and II (Fig. 7). However, we cannot rule out the possibility that the virus species undetectable in plasma are not completely eliminated from tissues, which might serve as a reservoir for generating future variants. In addition, other types of viruses may appear in other horses.

Most of the viruses belonging to the same type during the sequential episode had PND regions which predominantly consisted of different isoforms of the small units. The exceptions were the type II viruses (571FV, 571F2V, and 571F3V) obtained during the first three episodes in a 493F1V-infected horse (571); these viruses contained identical PND sequences. This seems to be due to lower levels of host immune responses to these viruses. Alternatively, the predominant host immune responses may have been directed to other regions, including other variable regions associated with the Env protein.

Horse 701 was immunized with V26 before V70 infection. Previous findings showed that V26 protected horses from challenge by cell-free V70 (13). Recently, we identified the specific insertion in the long terminal repeat of V26 compared to V70 (unpublished data). All the EIAVs derived from horse 701 during clinical episodes were found to be of the V70 type by RT-PCR (data not shown). Therefore, we included the sequence data obtained with the V70 variants from this horse in this study.

Duplications/insertions in other virus genomes have been observed. One group reported insertions/duplications of 3 to 27 nucleotides in the *env* V1 region and a 22-bp duplication in the long terminal repeat of simian immunodeficiency virus (36). Two other groups reported duplications in the *nef* gene of HIV-1 (3, 41). The sequences and structures of influenza virus proteins, characteristic of late evolution, also revealed that most have arisen through a process of gene duplication and recombination in some cases (4). Because of the complex interaction of primate lentiviruses with the immune system, immune-mediated selection for more pathogenic variants may be important. Under such selection, PND region duplications might occur in order to produce neutralization-resistant escape variants. Thus, EIAV could be a good model for characterizing the mechanisms of escaped variant lentivirus production.

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REFERENCES

1. **Alexandersen, S., and S. Carpenter.** 1991. Characterization of variable regions in the envelope and S3 open reading frame of equine infectious anemia virus. J. Virol. **65:**4255–4262.
2. **Ball, J. M., K. E. Rushlow, C. J. Issel, and R. C. Montelaro.** 1992. Detailed mapping of the antigenicity of the surface glycoprotein of equine infectious

anemia virus by using synthetic peptide strategies. J. Virol. **66:**732–742.
3. **Blumberg, B. M., L. G. Epstein, Y. Saito, D. Chen, L. R. Sharer, and R. Anand.** 1992. Human immunodeficiency virus type 1 *nef* quasispecies in pathological tissue. J. Virol. **66:**5256–5264.
4. **Brenner, S. E., T. Hubbard, A. Murzin, and C. Chotia.** 1995. Gene duplications in *H. influenzae*. Nature **378:**148.
5. **Cao, Y., D. D. Ho, J. Todd, R. Kokka, M. Urdea, J. D. Lifson, M. Piatak, Jr., S. Chen, B. H. Hahn, M. S. Saag, and G. M. Shaw.** 1995. Clinical evaluation of branched DNA signal amplification for quantifying HIV type 1 in human plasma. AIDS Res. Hum. Retroviruses **11:**353–361.
6. **Coffin, J. M.** 1996. Retroviridae: the viruses and their replication, p. 1767–1847. *In* B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Meinick, T. P. Monath, and B. Roizman (ed.), Virology, 3rd ed., vol. 2. Raven Press, New York, N.Y.
7. **Embretson, J., M. Zupancic, J. Beneke, M. Till, S. Wolinsky, J. L. Ribas, A. Burke, and A. T. Haase.** 1993. Analysis of human immunodeficiency virus-infected tissues by amplification of in situ hybridization reveals latent and permissive infections at single-cell resolution. Proc. Natl. Acad. Sci. USA **90:**357–361.
8. **Embretson, J., M. Zupancic, J. L. Ribas, A. Burke, P. Racz, R. K. Tenner, and A. T. Haase.** 1993. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. Nature **362:** 359–362.
9. **Goff, S. P.** 1990. Retroviral reverse transcriptase: synthesis, structure, and function. J. Acquired Immune Defic. Syndr. **3:**817–831.
10. **Hussain, K. A., C. J. Issel, K. L. Schnorr, P. M. Rwambo, and R. C. Montelaro.** 1987. Antigenic analysis of equine infectious anemia virus (EIAV) variants by using monoclonal antibodies: epitopes of glycoprotein gp90 of EIAV stimulate neutralizing antibodies. J. Virol. **61:**2956–2961.
11. **Kishi, M., Y.-H. Zheng, M. K. Bahmani, K. Tokunaga, H. Takahashi, M. Kakinuma, P. L. Lai, M. Nonoyama, R. B. Luftig, and K. Ikuta.** 1995. Naturally occurring accessory gene mutations lead to persistent human immunodeficiency virus type 1 infection of CD4-positive T cells. J. Virol. **69:**7507–7518.
12. **Kono, Y., and K. Kobayashi.** 1970. Changes in pathogenicity of equine infectious anemia virus during passages in horse leukocyte cultures. Natl. Inst. Anim. Health Q. **10:**106–112.
13. **Kono, Y., K. Kobayashi, and Y. Fukunaga.** 1970. Immunization of horses against equine infectious anemia (EIA) with an attenuated EIA virus. Natl. Inst. Anim. Health Q. **10:**113–122.
14. **Kono, Y., K. Kobayashi, and Y. Fukunaga.** 1973. Antigenic drift of equine infectious anemia virus in chronically infected horses. Arch. Virol. **41:**1–10.
15. **Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho.** 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J. Virol. **68:**4650–4655.
16. **Lichtenstein, D. L., C. J. Issel, and R. C. Montelaro.** 1996. Genomic quasispecies associated with the initiation of infection and disease in ponies experimentally infected with equine infectious anemia virus. J. Virol. **70:** 3346–3354.
17. **Meyerhans, A., R. Cheynier, J. Albert, M. Seth, S. Kwok, J. Sninsky, L. Morfeldt-Månson, B. Asjö, and S. Wain-Hobson.** 1989. Temporal fluctuations in HIV quasispecies in vivo are not reflected by sequential HIV isolations. Cell **58:**901–910.
18. **Michael, N. L., T. Mo, A. Merzouki, M. O'Shaughnessy, C. Oster, D. S. Burke, R. R. Redfield, D. L. Birx, and S. A. Cassol.** 1995. Human immunodeficiency virus type 1 cellular RNA load and splicing patterns predict disease progression in a longitudinally studied cohort. J. Virol. **69:**1868–1877.
19. **Montelaro, R. C., B. Parekh, A. Orrego, and C. J. Issel.** 1984. Antigenic variation during persistent infection by equine infectious anemia virus, a retrovirus. J. Biol. Chem. **259:**10539–10544.
20. **Nakaya, T., K. Fujinaga, H. Doi, S. Suzuki, H. Takahashi, Y. Nishino, M. Kishi, I. Azuma, R. B. Luftig, and K. Ikuta.** 1996. Serial passage of human immunodeficiency virus type 1 generates misalignment deletions in non-essential accessory genes. Virus Res. **46:**139–147.
21. **Nishimura, M., and H. Nakajima.** 1984. Structural proteins of equine infectious anemia virus and their antigenic activity. Am. J. Vet. Res. **45:**5–10.
22. **Palaniappan, C., M. Wisniewski, W. Wu, P. J. Fay, and R. A. Bambara.** 1996. Misincorporation by HIV-1 reverse transcriptase promotes recombination via strand transfer synthesis. J. Biol. Chem. **271:**22331–22338.
23. **Panteleo, G., J. F. Demarest, H. Soudeyns, J. W. Adelsberger, P. Borrow, M. S. Saag, G. M. Shaw, R. P. Sekaly, and A. S. Fauci.** 1994. Major expansion of $CD8^+$ T cells with a predominant Vβ usage during the primary immune response to HIV. Nature **370:**463–467.
24. **Panteleo, G., C. Graziosi, J. F. Demarest, L. Butini, M. Montroni, C. Fox, J. M. Orenstein, D. P. Kotler, and A. S. Fauci.** 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature **362:**355–358.
25. **Pathak, V. K., and H. M. Temin.** 1990. Broad spectrum of *in vivo* forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single replication cycle: deletions and deletions with insertions. Proc. Natl. Acad. Sci. USA **87:**6024–6028.
26. **Payne, S., K. Rushlow, B. Dhruva, C. Issel, and R. Montelaro.** 1989. Localization of conserved and variable antigenic domains of equine infectious anemia virus envelope glycoproteins using recombinant *env*-encoded protein fragments produced in *Escherichia coli*. Virology **172:**609–615.
27. **Payne, S. L., F.-D. Fang, C. P. Liu, B. R. Dhruva, P. Rwambo, C. J. Issel, and R. C. Montelaro.** 1987. Antigenic variation lentivirus persistence: variations in envelope gene sequences during EIAV infection resemble changes reported for sequential isolates of HIV. Virology **161:**321–331.
28. **Payne, S. L., B. Parekh, R. C. Montelaro, and C. J. Issel.** 1984. Genomic alterations associated with persistent infections by equine infectious anemia virus. J. Gen. Virol. **65:**1395–1399.
29. **Payne, S. L., O. Salinovich, S. Nauman, C. Issel, and R. Montelaro.** 1987. Course and extent of variation of equine infectious anemia virus during parallel persistent infections. J. Virol. **61:**1266–1270.
30. **Perelson, A. S., A. U. Neumann, M. Markowitz, J. M. Leonard, and D. D. Ho.** 1996. HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time. Science **271:**1582–1586.
31. **Pulsinelli, G. A., and H. M. Temin.** 1991. Characterization of large deletions occurring during a single round of retrovirus vector replication: novel deletion mechanism involving errors in strand transfer. J. Virol. **65:**4786–4797.
32. **Ricchetti, M., and H. Buc.** 1990. Reverse transcriptases and genomic viability: the accuracy of DNA replication is enzyme specific and sequence dependent. EMBO J. **9:**1583–1593.
33. **Roberts, J. D., K. Bebenek, and T. A. Kunkel.** 1988. The accuracy of reverse transcriptase from HIV-1. Science **242:**1171–1173.
34. **Salinovich, O., S. L. Payne, R. C. Montelaro, K. A. Hussain, C. J. Issel, and K. L. Schnorr.** 1986. Rapid emergence of novel antigenic and genetic variants of equine infectious anemia virus during persistent infection. J. Virol. **57:**71–80.
35. **Sellon, D. C., F. J. Fuller, and T. C. McGuire.** 1994. The immunopathogenesis of equine infectious anemia virus. Virus Res. **32:**111–138.
36. **Tao, B., and P. N. Fultz.** 1995. Molecular and biological analyses of quasispecies during evolution of a virulent simian immunodeficiency virus, SIVsmmPBj14. J. Virol. **69:**2031–2037.
37. **Temin, H. M.** 1993. Retrovirus variation and reverse transcription: abnormal strand transfers result in retrovirus genetic variation. Proc. Natl. Acad. Sci. USA **90:**6900–6903.
38. **Varmus, H., and P. Brown.** 1989. Retroviruses, p. 53–108. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
39. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, H. H. Hahn, M. S. Saag, and G. M. Shaw.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature **373:**117–122.
40. **Zheng, Y. H., T. Nakaya, H. Sentsui, M. Kameoka, M. Kishi, K. Hagiwara, H. Takahashi, Y. Kono, and K. Ikuta.** 1997. Insertions, duplications and substitutions in restricted gp90 regions of equine infectious anemia virus occurred during febrile episodes in an experimentally infected horse. J. Gen. Virol. **78:**807–820.
41. **Zhong, Q., T. Nakaya, Y. Tateno, K. Fujinaga, M. Kameoka, M. Tateno, and K. Ikuta.** A clearer distinction between HIV-1 paired isolates from peripheral blood mononuclear cells of asymptomatic carriers with and without $CD8^+$ T-cells at *nef* rather than *env* V3 loci. Vaccine, in press.