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Rapid Emergence of Novel Antigenic and Genetic Variants of Equine Infectious Anemia Virus during Persistent Infection

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Previous results from our laboratory have demonstrated that equine infectious anemia virus displays structural variations in its surface glycoproteins and RNA genome during passage and chronic infections in experimentally infected Shetland ponies (Montelaro et al., J. Biol. Chem. 259:10539–10544, 1984; Payne et al., J. Gen. Virol. 65:1395–1399, 1984). The present study was undertaken to obtain an antigenic and biochemical characterization of equine infectious anemia virus isolates recovered from an experimentally infected pony during sequential disease episodes, each separated by intervals of only 4 to 8 weeks. The virus isolates could be distinguished antigenically by neutralization assays with serum from the infected pony and by Western blot analysis with a monoclonal antibody against the major surface glycoprotein gp90, thus demonstrating that novel antigenic variants of equine infectious anemia virus predominate during each clinical episode. The respective virion glycoproteins displayed different electrophoretic mobilities on sodium dodecyl sulfate-polyacrylamide gels, indicating structural variation. Tryptic peptide and glycopeptide maps of the viral proteins of each virus isolate revealed biochemical alterations involving amino acid sequence and glycosylation patterns in the virion surface glycoproteins gp90 and gp45. In contrast, no structural variation was observed in the internal viral proteins pp15, p26, and p9 from any of the four virus isolates. Oligonucleotide mapping experiments revealed similar but unique RNase T1-resistant oligonucleotide fingerprints of the RNA genomes of each of the virus isolates. Localization of altered oligonucleotides for one virus isolate placed two of three unique oligonucleotides within the predicted *env* gene region of the genome, perhaps correlating with the structural variation observed in the envelope glycoproteins. Thus these results support the concept that equine infectious anemia virus is indeed capable of relatively rapid genomic variations during replication, some of which result in altered glycoprotein structures and antigenic variants which are responsible for the unique periodic disease nature observed in persistently infected animals. The findings of envelope specific differences in isolates of visna virus and of human T-cell lymphotropic virus III (acquired immune deficiency syndrome-related virus) suggest that this variation may be a common characteristic of the subfamily Lentivirinae.

During recent years, the capacity and frequency of RNA virus variation and the biological significance of this variation in persistent infections has become increasingly evident in systems such as poliovirus, vesicular stomatitis virus, and influenza virus (18). Among retroviruses, where provirus establishment has previously been regarded as the primary mechanism of viral persistence, antigenic variation has been demonstrated for visna virus by neutralization assays, peptide mapping of viral antigens, and oligonucleotide mapping and sequencing of viral genomes (8, 35, 48). The results of these studies indicate specific variation in the virion glycoprotein gp135 resulting from point mutations in the viral *env* gene. However, the relevance of this antigenic variation to viral persistence has recently been questioned by results of serological studies which report the continued presence of parental virus strains long after the emergence of new visna virus variants in experimentally infected sheep (25), and by the lack of antigenic variants even during advanced stages of disease (52).

Recently, we have reported the occurrence of antigenic variation in the retrovirus-induced disease equine infectious anemia (31, 41). This antigenic variation apparently explains the unique periodic nature of the disease and the inefficacy of classical vaccination protocols in providing protection. In previous studies from our laboratory, we demonstrated that selected virus isolates from different clinical episodes in experimentally infected ponies can be distinguished antigenically in neutralization assays. Moreover, peptide mapping studies demonstrated significant structural changes in the polypeptide portion of the major envelope glycoprotein gp90 from antigenically distinct virus isolates, whereas the structures of internal proteins remained constant (31). Oligonucleotide mapping studies determined that structural variation could also be observed at the level of the viral genome and that point mutations were the probable mechanism by which this variation occurred (41). However, these initial studies were limited in several ways. The isolates examined were from nonconsecutive clinical episodes separated by long periods of time (3 to 6 months), and comparisons of tryptic peptide maps of the second glycoprotein of equine infectious anemia virus (EIAV), gp45, were not technically possible. Also, the two-dimensional mapping procedure used to separate tryptic peptides was not able to adequately resolve glycosylated peptides for a comparative analysis.

The present investigation was undertaken to extend our examination of antigenic variation by analyzing four virus isolates recovered from consecutive disease episodes 4 to 8 weeks apart in a single experimentally infected pony. Neutralization assays with serum samples from the infected pony were performed to ensure that these EIAV isolates could be

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distinguished antigenically. In addition, monoclonal antibody was employed to analyze antigenic variation in a single gp90 epitope in each virus strain. As in the previous studies, gp90 as well as the internal proteins p26, pp15, and p9 from these four virus isolates were subjected to tryptic peptide mapping. The gp45 component of these isolates was for the first time successfully mapped, thus allowing the examination of both surface glycoprotein antigens. Also, a modification of the tryptic peptide mapping procedure made it possible to examine the glycosylated peptides from both gp90 and gp45. Oligonucleotide mapping studies were conducted for the same four isolates to determine whether structural alterations in the RNA genome could be detected between virus strains recovered only weeks apart. Furthermore, localization studies were performed to map oligonucleotides of interest to a specific region of the genome. The results of these studies provide a detailed biochemical and serological characterization of uniquely rapid variations in EIAV during persistent infections in ponies.

MATERIALS AND METHODS

Virus isolates and assays. The prototype stock of EIAV was obtained by propagation in fetal equine kidney (FEK) cells (30) of the Wyoming cell-adapted strain of EIAV (27). This prototype strain of virus was then used in experimental serial passages in Shetland ponies to establish the recurrent febrile episodes characteristic of equine infectious anemia (39; A. Orrego, Ph.D. thesis, Louisiana State University, Baton Rouge, 1983). Figure 1 summarizes the clinical history of the experimentally infected pony in the third serial passage, from which virus isolates used in these studies were recovered. Each isolate was obtained from a plasma sample taken during the indicated disease episode and purified by endpoint dilution in FEK cells to ensure recovery of the predominant virus population (31). Endpoint dilutions were used to purify all EIAV isolates due to the lack of plaque or other cytopathic assays for this virus. No virus was recovered from plasma samples taken during afebrile periods in this animal, confirming previous observations that plasma viremia is usually restricted to disease episodes (39).

Virus production was monitored by a p26-specific enzyme-linked immunosorbent assay as described previously (39, 49). Virus infectivity was expressed in terms of $\log_{10}$ 50% tissue culture infectious doses per 0.5-ml sample, and serum neutralization tests were carried out by the virus dilution-constant serum method (31). Serum neutralizing activity was expressed as a $\log_{10}$ neutralizing index, calculated as the difference in $\log_{10}$ titer of the virus samples with or without test serum. A neutralizing index of at least 1.5 was considered positive. The neutralization index method was employed in these studies because of the absence of a constant virus-serum variation assay for EIAV. In addition, the neutralization index method conserves the limited amounts of virus and serum available.

Virus samples used for protein analysis were obtained from roller bottle cultures of infected FEK cells and purified by equilibrium density centrifugation on glycerol gradients (30, 31, 40).

Glycoprotein purification. Procedures for EIAV glycoprotein purification by lentil lectin chromatography have been described and evaluated previously (31, 32). Typically, approximately 100 to 150 μg of glycoproteins was recovered from 4 mg of whole virus by using these procedures.

Production of monoclonal antibodies. BALB/c mice were immunized subcutaneously and intraperitoneally with 100 μg of lentil lectin-purified glycoproteins from prototype EIAV in Freund complete adjuvant. Mice were boosted on day 14 with antigen preparations in Freund incomplete adjuvant and again on days 28 and 88 with antigen in 0.01 M phosphate buffer. Four days after the final injection, spleens from immunized mice were removed and minced, and the cells were fused with SP2/0 mouse myeloma cells as described by Groth and Schneidegger (15). Hybridomas were cloned on HAT medium (15) and then screened for EIAV-specific antibody secretion by the solid-phase enzyme-linked immunosorbent assay technique described previously (49). Ascites fluids were produced by intraperitoneal injection of 1×10^6 to 5×10^6 hybridoma cells into syngeneic mice previously injected with pristane (28).

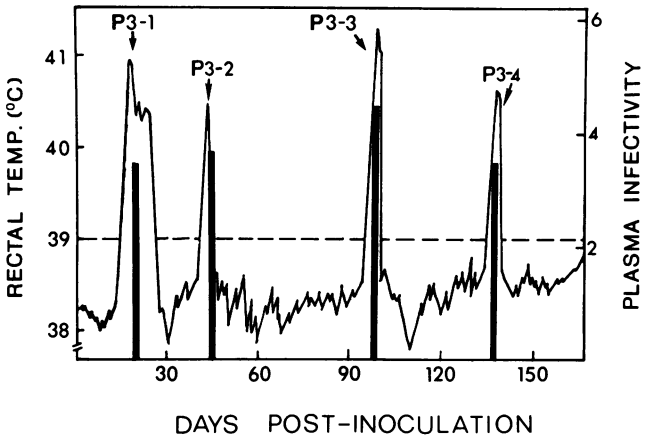

FIG. 1. Clinical history of an experimentally infected pony showing disease episodes from which virus isolates were recovered. All sustained rectal temperature recordings above 39°C are considered abnormal and designated as febrile episodes. Vertical bars indicate plasma viremia levels at the time of isolate recovery, as determined by titration on fetal equine kidney cells, and expressed as $\log_{10}$ 50% tissue culture infectious doses per 0.5 ml (39).

SDS-PAGE and immunoblots. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of protein pools for peptide mapping experiments was performed on 10 or 15% polyacrylamide gels as described by Laemmli (24). Protein bands were visualized by staining with Coomassie brilliant blue for 30 min, followed by immediate destaining with multiple changes of methanol-acetic acid-water (10:3:27). To identify immunoreactive components, viral proteins were resolved by SDS-PAGE as described by Montelaro et al. (30), transferred to nitrocellulose membranes, and immunoblotted with either reference serum from a naturally infected horse or ascites fluids from monoclonal preparations, as described by Burnette et al. (6). In the case of the ascites immunoblots, this procedure was modified to include the addition of goat anti-mouse immunoglobulin G (IgG) before incubation with ^{125}I-labeled protein A, since protein A binds very weakly, if at all, to certain mouse IgG subclasses (4, 22, 26).

Peptide and glycopeptide mapping. Peptide mapping of proteins separated by SDS-PAGE was performed by in situ radioiodination and trypsin digestion, followed by two-dimensional thin-layer chromatographic analysis of labeled tryptic peptides (11, 12, 31).

As shown previously, only nonglycosylated peptides can be analyzed effectively by this two-dimensional thin-layer chromatographic procedure, since glycosylated peptides fail to migrate in the second-dimension chromatography (31).

TABLE 1. Serum neutralization indices of sera collected at different times after inoculation

Virus strain	VID[a]	Neutralization index[b] of serum collected on the following day postinoculation:		
		31	125	158
P3-1	13	1.5	3.0	3.0
P3-2	41	0.5	3.0	2.5
P3-3	99	1.5	2.0	2.5
P3-4	137	1.0	1.0	1.0

[a] Days postinoculation when plasma was collected.
[b] Log_{10} serum neutralization index (positive if ≥1.5) as described in Materials and Methods.

Therefore, the mapping procedure above was modified for the analysis of tryptic glycopeptides (O. Salinovich and R. C. Montelaro, Anal. Biochem., in press). Briefly, glycosylated peptides were separated from the nonglycosylated peptides of tryptic digests of radioiodinated glycoproteins by lentil lectin affinity chromatography. Glycopeptides (lectin binding) were then dialyzed against 0.05 M ammonium bicarbonate in Spectrapore 3 dialysis tubing, which had been boiled for 15 min in 0.01 M EDTA to reduce the effective pore size such that peptides of molecular weight greater than 700 would be retained. The dialyzed glycopeptide fraction was then lyophilized to dryness, dissolved in 15 μl of electrophoresis solvent (acetic acid-formic acid-water, 15:5:80), and separated on 20- by 20-cm cellulose-coated thin-layer chromatography plates by high-voltage electrophoresis as performed for peptide mapping, except that the migration distance of the tracking dye was increased to 15 cm. Chromatography was then performed for 10 h in a perpendicular direction in a modified solvent consisting of 1-butanol–pyridine–acetic acid–water (5:5:1:7.5). The separated radioiodinated glycopeptides were detected by autoradiography with Kodak X-Omat film (XRP-5).

Oligonucleotide mapping. Intact genomic RNA was isolated from purified EIAV as described previously (41). Briefly, culture medium from infected FEK cells was collected at 12-h intervals and clarified of cellular debris by centrifugation at 13,000 × *g* for 20 min at 4°C. Virus was then pelleted by centrifugation (36,000 × *g* for 2 h at 4°C) through 10% sucrose in TNE buffer (10 mM Tris chloride [pH 8.0], 100 mM NaCl, 1 mM EDTA). The virus pellets were suspended in TNE buffer and pelleted again by centrifugation through 20% sucrose at 190,000 × *g* for 1 h at 4°C. The resultant virus pellet was then suspended in TNE buffer and digested with proteinase K (0.1 mg/ml) in 0.2% SDS for 15 min at 25°C. The protease-treated virus RNA was extracted once with phenol-chloroform (1:1) and once with chloroform-isoamyl alcohol (24:1) and then purified by velocity sedimentation (190,000 × *g* for 1.75 h at 4°C) through 10 to 30% sucrose in TNE buffer (23, 34). Fractions containing 70S RNA were pooled and concentrated by ethanol precipitation.

For oligonucleotide mapping, purified 70S RNA (400 to 500 ng) was digested with RNase T1 in the presence of bacterial alkaline phosphatase, and the resultant oligonucleotides were terminally labelled with [γ-^{32}P]ATP (ICN Pharmaceuticals) as described by Pederson and Haseltine (42, 43). The radiolabeled oligonucleotides were separated by two-dimensional gel electrophoresis (37) followed by autoradiography. For each virus isolate, RNA was purified and digested on at least two separate occasions, and three or four maps were used in making comparisons between any two isolates. Because of the high reproducibility of the maps obtained by these procedures (41–43), comparisons were performed by directly overlaying autoradiographs. Thus, deletions and additions of oligonucleotides are based on their characteristic migration position in the map. No further attempts were made in these studies to determine the identity of matching oligonucleotides between virus isolates.

Oligonucleotide localization studies. The oligonucleotides of a selected EIAV isolate (P3-3) were localized within the viral genome by using a strategy described previously for other retroviruses (3, 8). This technique involves oligonucleotide mapping of subgenomic RNA fragments which lack various amounts of the 5′ end of the viral genome. Virus and 70S genomic RNA were prepared as described above, except that virus was harvested at 24- to 48-h intervals to recover naturally fragmented RNA genomes. Poly(A)-containing RNA was isolated by chromatography over oligo(dT) cellulose (Collaborative Research, Lexington, Mass.) as described by Aviv and Leder (2), and genomic RNA fragments of different sizes were separated by sedimentation (200,000 × *g* for 6 h at 20°C) through linear gradients of 10 to 30% sucrose in TNE buffer with 0.1% SDS. A gradient containing 18S and 28S rRNA as size markers was run in parallel. Viral RNA fragments of selected sizes ranging from 5S to 35S were recovered from the sucrose gradient by ethanol precipitation. Oligonucleotide mapping of the different subgenomic fragments was performed as described above. Localization of oligonucleotides to the 3′ end of the genome was performed by visual comparison of maps of full-length viral RNA with maps of RNA fragments lacking approximately one-half to three-fourths of the total genome, as estimated by sedimentation values.

RESULTS

Neutralization studies. The present study involves four EIAV isolates recovered from a single experimentally infected pony, with each febrile episode separated by only 4 to 8 weeks (Fig. 1). The neutralization data contained in Table 1 indicate that serum taken 18 days after the appearance of the first disease episode displayed a log_{10} neutralization index of 1.5 against P3-1, but was negative against isolates P3-2 and P3-4. A positive neutralizing activity was also observed against P3-3. However, serum taken 26 days after the isolation of P3-3 displayed a neutralization index of 2.0 or greater when tested against P3-3 or previous isolates, but was negative against P3-4. Serum collected 21 days after the isolation of P3-4 displayed a neutralization index of 2.5 or greater against P3-1 through P3-3, but failed to neutralize isolate P3-4. Apparently, significant neutralizing antibody against P3-4 was not yet present, and unfortunately the animal expired approximately 10 days later, before a final serum sample was obtained.

These results indicate that distinct antigenic variants of EIAV are associated with each disease episode in the sequential series, thus confirming and extending our previous report (31). Moreover, the data demonstrate that the time required for EIAV to vary its antigenic reactivity such that it can escape host immune defenses can be as short as 28 days (the time between the recovery of isolates P3-1 and P3-2). Thus, the time frame required for antigenic variation in EIAV is considerably shorter than that reported for visna virus, which requires 1 to 2 years for the appearance of antigenically distinct virus strains in the persistently infected animal (8, 48).

Immunoblot analyses. Immunoblot analysis of previously characterized EIAV isolates demonstrated variations in electrophoretic mobility of the respective envelope glycoproteins gp90 and gp45 (31). Therefore, samples of the four consecutive virus isolates P3-1 through P3-4 were separated by SDS-PAGE and probed with our reference equine immune serum, which exhibits a broad spectrum of reactivity. Figure 2A shows the results of such an immunoblot experiment comparing all four virus isolates and the prototype EIAV strain. Variations in electrophoretic mobility of the gp90 and gp45 components from the individual isolates were observed, suggesting structural differences in the respective glycoproteins of the EIAV isolates. In contrast, the migration rate of the internal protein p26 from all five virus strains remained constant. Some variation in the degree of reactivity of the reference antiserum to the glycoproteins of the different isolates was also observed; the serum displayed a very poor reactivity to the gp45 of isolate P3-3, whereas it reacted strongly with the gp45 from the remaining isolates.

To determine whether subtle antigenic differences not detectable with a broadly reactive reference serum existed between the four virus isolates, a similar blotting analysis with a monoclonal antibody produced against gp90 from prototype EIAV was performed (Fig. 2B). As expected, this monoclonal antibody reacted very strongly with gp90 from prototype virus. It also reacted equally well with P3-2 gp90 and recognized the P3-4 glycoprotein to a lesser extent. However, it displayed only negligible reactivity with gp90 from P3-1 and completely failed to recognize P3-3 gp90. This monoclonal antibody is apparently specific for an epitope of the gp90 molecule which undergoes structural changes during persistent infection. These results demonstrate for the first time that definitive antigenic changes at a single epitope have occurred in the gp90 molecule and that these changes have occurred over a relatively short period of time (e.g., P3-2 to P3-3). However, it is not certain whether the variant epitope identified by this monoclonal antibody is critical for virus neutralization, since the test monoclonal antibody did not neutralize the prototype strain or other isolates of EIAV in preliminary neutralization assays (data not shown).

Peptide and glycopeptide mapping. To define further the structural changes suggested by the SDS-PAGE and immunoblotting analyses, virion glycoproteins purified by lentil lectin chromatography and separated by SDS-PAGE were iodinated and trypsinized in situ and analyzed by two-dimensional mapping procedures. Peptide maps of the gp90 components from the four virus isolates P3-1 through P3-4 are shown in Fig. 3A through D, and maps obtained of the respective gp45 components are shown in Fig. 4A through D. To obtain a systematic analysis, peptide additions and deletions are indicated based on a comparison of each strain with its immediate predecessor. In this manner, P3-2 is compared with P3-1, P3-3 is compared with P3-2, and P3-4 is compared with P3-3. The results of each comparison of the gp90 peptide maps reveal differences in each pair of isolates examined. Similarly, there are peptide additions and deletions observed in the gp45 maps for each pair of virus isolates. These comparisons demonstrate for the first time that structural changes occur in both glycoproteins of EIAV during the course of a single persistent infection. Three to five maps of each virus isolate were studied, and the denoted differences were consistently observed in all maps. Also, the maps obtained were completely reproducible, even after passage of the virus strains in cell culture for as long as 6 months.

The glycosylation patterns of the glycoproteins of the EIAV isolates were compared by using a two-dimensional procedure modified for the resolution of glycosylated peptides purified by lectin affinity chromatography (Salinovich and Montelaro, in press). The maps obtained for glycosylated tryptic peptides of the gp90 component from each isolate are shown in Fig. 3E through H, and glycopeptide maps of the respective gp45 components are shown in Fig. 4E through H. These maps reveal the existence of glycopeptide "families" which possess identical electrophoretic mobilities, but differ in their mobility in the second dimension chromatography solvent, as observed previously for well-characterized glycoproteins such as α_1-acid glycoprotein or fetuin (Salinovich and Montelaro, in press). This pattern in glycopeptide maps is possibly due to microheterogeneity within the carbohydrate moiety, which has been demonstrated in these model glycoproteins (36, 47). Therefore, the absence or presence of entire glycopeptide families, as opposed to individual glycopeptides, was considered in all glycopeptide map comparisons.

Although minor differences can be observed within the same glycopeptide family from different isolates, there appear to be only two distinct patterns of glycosylation exhibited by gp90 from the isolates studied. Strains P3-1, P3-2, and P3-4 possess one of these patterns, whereas P3-3 exhibits a strikingly different pattern. Similar results were observed in the gp45 glycopeptide maps, which are less complex than those of gp90. As with the larger glycoprotein, there appear to be only two distinct glycosylation patterns for the gp45 component from each isolate. These patterns are exhibited in an alternating fashion, one by P3-1 and P3-3 and the other by P3-2 and P3-4. This recurrence of glycopeptide patterns between any two isolates is in contrast

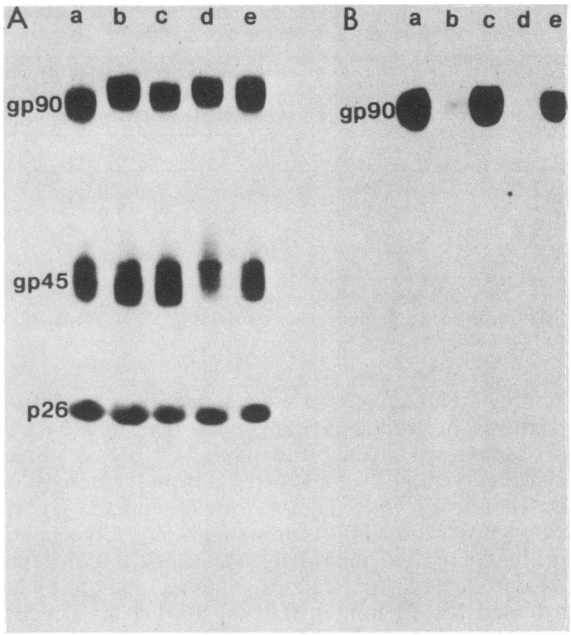

FIG. 2. Immunoblot analysis of purified test strains of EIAV: prototype (a), P3-1 (b), P3-2 (c), P3-3 (d), and P3-4 (e). (A) Samples of 70 μg of each virus isolate were analyzed by SDS-PAGE, transferred to nitrocellulose, and treated with reference horse immune serum to identify the immunogenic virion proteins. (B) Samples of 50 μg of each virus isolate were analyzed by SDS-PAGE and transferred as in panel A and then treated with a monoclonal antibody generated against gp90 from prototype EIAV.

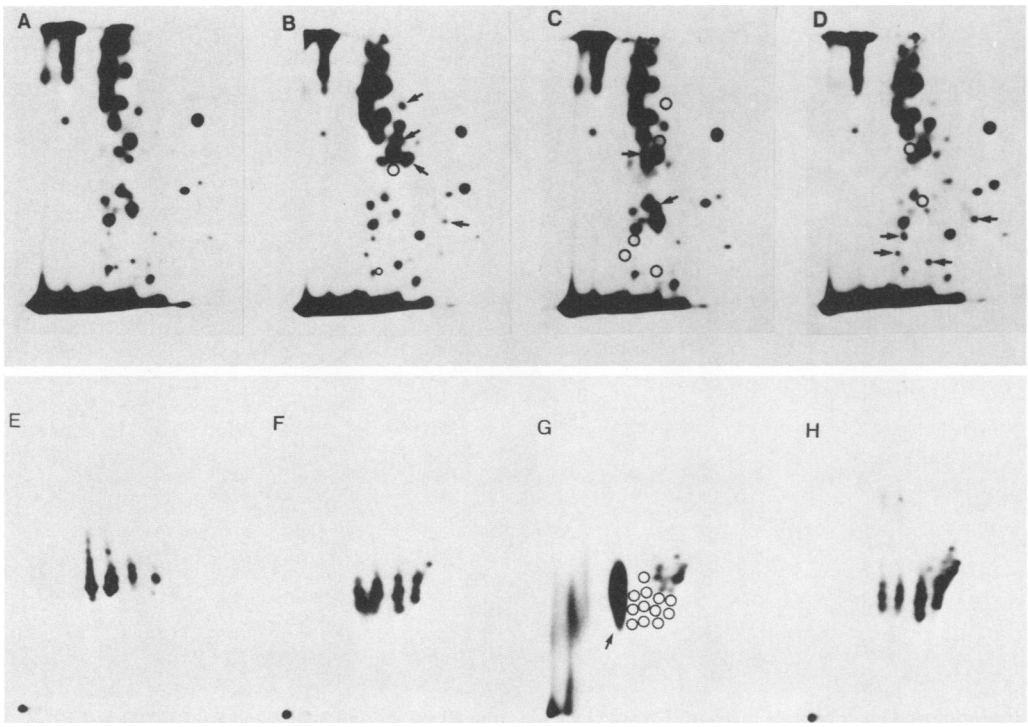

FIG. 3. Tryptic peptide and glycopeptide maps of ^{125}I-gp90 isolated from EIAV strain P3-1 (A and E), P3-2 (B and F), P3-3 (C and G), and P3-4 (D and H). The two-dimensional analysis consisted of electrophoresis in the horizontal direction and chromatography in the vertical direction. Samples were applied in the lower left corner. Panels A through D show maps of nonglycosylated peptides of gp90 from each isolate. Arrows (additions) and circles (deletions) indicate peptide differences in each map when compared with that isolate's immediate predecessor. Thus P3-2 is compared with P3-1, P3-3 is compared with P3-2, and P3-4 is compared with P3-3. Panels E through H show maps of glycosylated peptides isolated from gp90 from each virus strain. Due to probable microheterogeneity of the carbohydrate moieties, vertical families of glycopeptides which possess the same electrophoretic mobility were utilized in comparisons instead of individual glycopeptides. Arrows (additions) and circles (deletions) in panel G indicate major differences in this map of P3-3 gp90 glycopeptides as compared with maps from the other three isolates.

to the peptide maps of gp90 and gp45, in which no two patterns are identical.

These results indicate that the surface glycoprotein antigens gp90 and gp45 both undergo rapid structural variations during persistent infection in a single animal, in correlation with the neutralization data described above. This variation is not restricted to the polypeptide portion of the molecule, since different patterns of glycosylation are also observed for these antigens from different virus isolates.

Peptide maps were also constructed from the respective p26, pp15, and p9 components of each virus isolate after separation by SDS-PAGE. The p11 component cannot be mapped by this method, because it contains no tyrosyl residues and therefore will not radioiodinate (30). Representative maps of p26 are shown in Fig. 5. There were no changes in the patterns obtained for this internal nonglycosylated structural protein in any of the isolates compared. A similar conservation of structure was indicated by the peptide maps of pp15 and p9 (data not shown), as reported previously (31). Thus the structural variation observed in EIAV during a persistent infection is evidently specific for the virion glycoproteins, which are subject to immune selection mechanisms.

Oligonucleotide mapping. RNase T1-resistant oligonucleotide maps were obtained for isolates P3-1 through P3-4 to characterize the level of genomic variation occurring during the model persistent infection. Comparison of these maps (Fig. 6) reveals highly similar but unique sets of oligonucleotides for each of the four isolates. The map of EIAV P3-2 compared with that of its immediate predecessor, P3-1, shows two oligonucleotide deletions (open circles in Fig. 6B). The oligonucleotide map for isolate P3-3 has three additions when compared to isolate P3-2, whereas that for P3-4 has two deletions and one addition when compared with its immediate predecessor, P3-3. It is interesting to note that, although a small number of oligonucleotides seem to be variable, no two maps are identical. Moreover, the designated differences between the oligonucleotide maps of the EIAV isolates were completely reproducible in multiple experiments and stable to passage in tissue culture for at least 6 months.

Genomic localization studies. To map variant oligonucleotides to a specific region of the viral genome, the oligonucleotides of EIAV isolate P3-3 were localized with respect to the 3′ end of the viral RNA. According to molecular weight estimates of virion polypeptides (40) and assuming that the organization of the EIAV genome is similar to that of other nononcogenic retroviruses (S. Oroszlan, personal communication), the size of the *env* gene can be estimated to approach half of the total genome and thus is expected to comprise approximately 4,000 nucleotides at the 3′ terminus of the viral RNA.

Isolate P3-3 was chosen for this study as it contains three altered oligonucleotides (additions) when compared with isolate P3-2. Two of these altered oligonucleotides are again deleted in isolate P3-4. Figure 7 shows an oligonucleotide

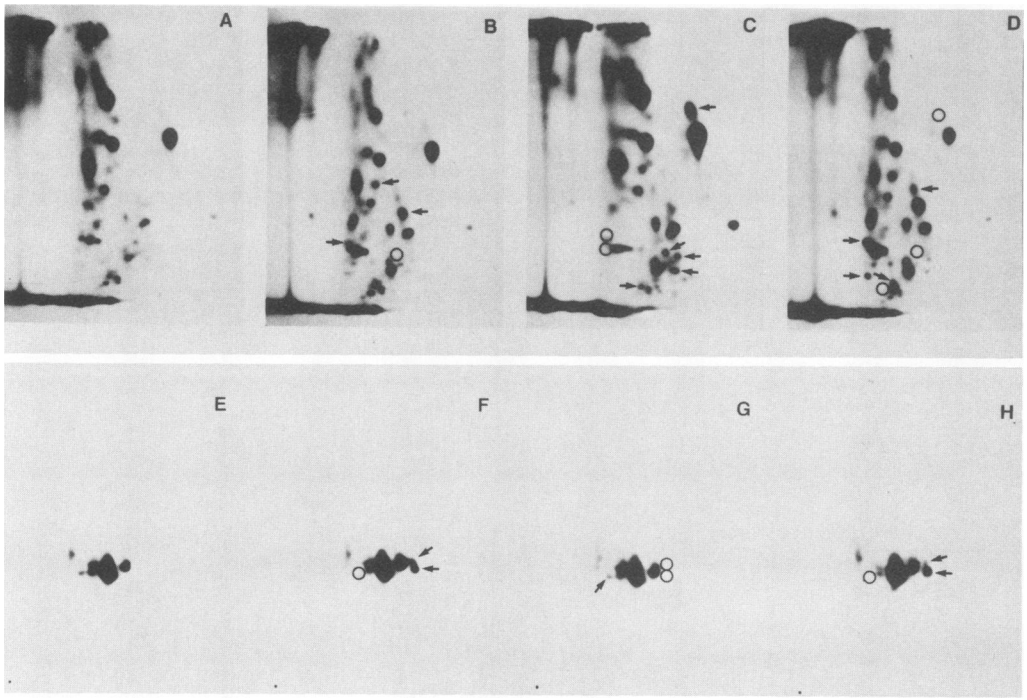

FIG. 4. Tryptic peptide and glycopeptide maps of ^{125}I-gp45 isolated from EIAV strain P3-1 (A and E), P3-2 (B and F), P3-3 (C and G), and P3-4 (D and H). Panels A through D show peptide analysis and comparisons as described in the legend to Fig. 3. In panels E though H, arrows (additions) and circles (deletions) represent glycopeptide differences in each map when compared to that isolate's immediate predecessor, as indicated for the gp45 peptide maps.

map of full length viral RNA, as well as maps of fragments lacking approximately one-third to three-fourths of the 5′ end of the total genome. The three additions seen previously in this isolate are clearly present in the map of full-length genomic RNA. The altered oligonucleotide designated a is not present in the map of 18S to 21S viral RNA, indicating that this oligonucleotide, although unique to some virus isolates, probably represents an alteration in the viral RNA in a region other than the *env* gene. Oligonucleotides b and c, however, are clearly present in Fig. 7B. These two oligonucleotides are still present in a map of 12S to 15S viral RNA, although the intensity of b is diminished. Many other oligonucleotides have completely disappeared, as would be expected since this RNA fragment is lacking over half of the total genome, assuming that decreases in S value reasonably estimate decreases in molecular weight. Only oligonucleotide c is still present in viral RNA fragments with sedimentation values of only 7S to 10S, indicating that the genomic alterations reflected by the presence or absence of this oligonucleotide are occurring in the 3′ end of the viral genome.

If the organization of the *env* gene is similar to that of other retroviruses such as murine leukemia virus (29), it may be assumed to code for a polyprotein containing gp90 at the amino terminus and gp45 at the carboxy terminus. If this is the case, then oligonucleotide b can be tentatively placed in the region of the *env* gene coding for gp90, and oligonucleotide c can be localized to gp45. Thus, these oligonucleotide changes may correlate with changes observed in the polypeptide portion of these two envelope glycoproteins, which in turn may be linked to the antigenic changes seen during persistent EIAV infection.

DISCUSSION

EIAV is a retrovirus usually assigned to the subfamily Lentivirinae, which is a group of nontransforming

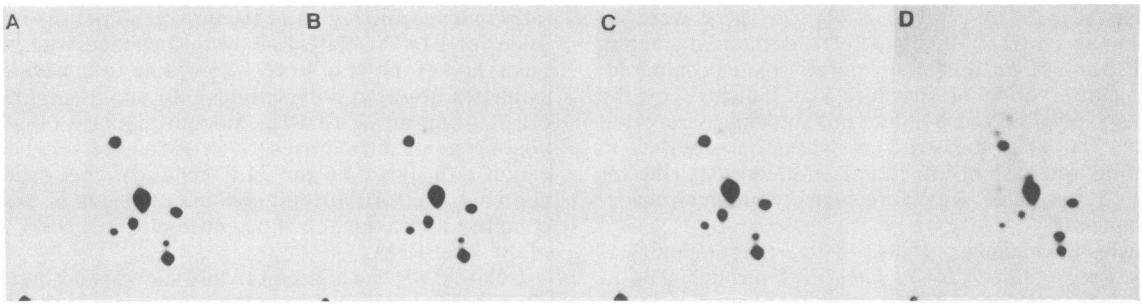

FIG. 5. Tryptic peptide maps of ^{125}I-p26 isolated from EIAV strain P3-1 (A), P3-2 (B), P3-3 (C), and P3-4 (D). Peptide analysis and comparisons are as described in the legend to Fig. 3.

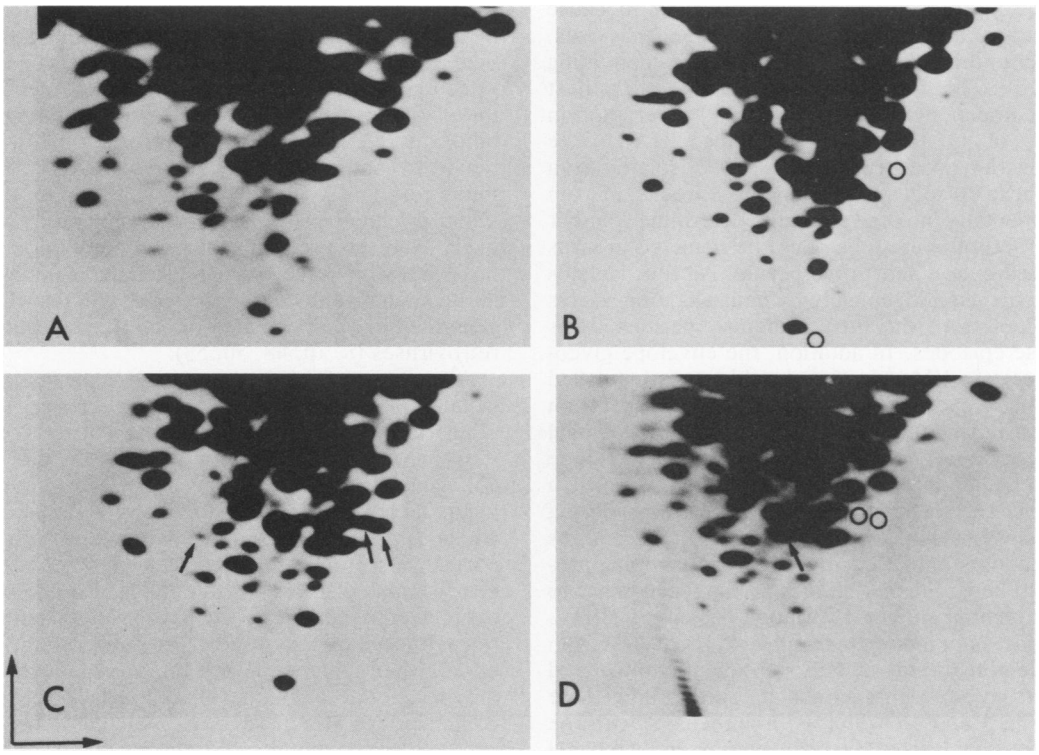

FIG. 6. Oligonucleotide maps of EIAV 70S genomic RNA from third-passage isolates P3-1 (A), P3-2 (B), P3-3 (C), and P3-4 (D). The direction for electrophoresis in the first dimension (8% polyacrylamide, pH 3.25) was from left to right, and the direction in the second dimension (23% polyacrylamide, pH 8.2) was from bottom to top. Arrows (additions) and open circles (deletions) indicate differences in each pattern when compared to its immediate predecessor, as was done in the peptide maps.

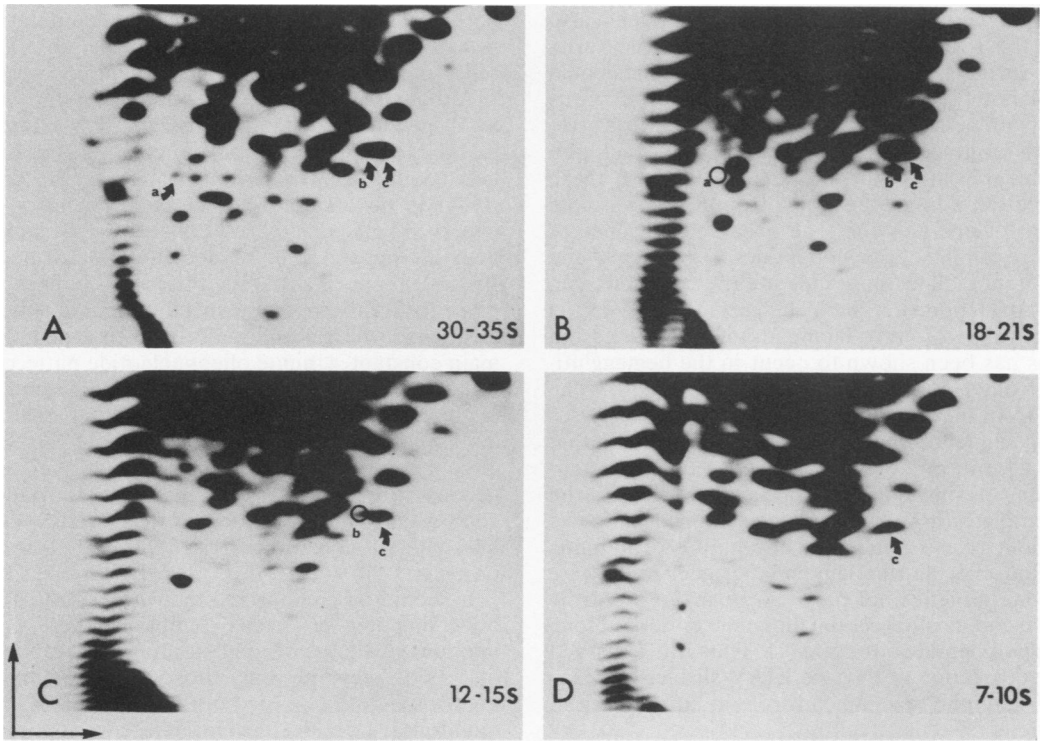

FIG. 7. Oligonucleotide maps of subgenomic, poly(A)-containing RNA from EIAV isolate P3-3. Subgenomic RNA was purified as described in the text. Oligonucleotides a, b, and c appeared as additions upon comparison of P3-2 with P3-3 in Fig. 6 whereas b and c were deletions upon comparison of P3-3 with P3-4. Autoradiographs of subgenomic RNA were compared visually for the absence or presence of oligonucleotides a, b, and c. Open circles indicate the positions of absent or diminished oligonucleotides.

exogenous viruses characterized by their ability to cause persistent infection in the host (7, 51). Like visna virus, another member of this subfamily which causes a debilitating disease in sheep (8, 14, 16, 48), EIAV is believed to persist in the face of competent immune responses by variation in the antigenicity of its surface glycoproteins (21, 31). The present investigations focused on four EIAV isolates from consecutive febrile episodes occurring 4 to 8 weeks apart in a single experimentally infected animal to determine whether antigenic or structural variations could be detected in virus strains recovered over a short time period. All four isolates could be distinguished antigenically by neutralization assays with serum taken from the host animal at various times between disease episodes. In addition, the envelope glycoproteins from certain EIAV isolates could be distinguished according to their reactivity with either a broad-spectrum reference antiserum in the case of gp45 or a monoclonal antibody in the case of gp90. Thus, it is clear that a unique predominant population of virus is associated with each episode. Whether certain minor populations also exist during these episodes is an important question which requires analytical procedures more sensitive than the mapping protocols employed here. Further studies are also necessary to determine the extent of antigenic variation possible in EIAV.

Tryptic peptide mapping of gp90 and gp45 recovered from each isolate demonstrated several peptide additions and deletions occurring between each isolate and its immediate predecessor (Fig. 3 and 4), which is in agreement with the results of previous analysis of the gp90 component of a panel of EIAV isolates (31). We have reported previously that approximately 10 to 15% of the host's natural immune response to gp90 is directed toward the oligosaccharides present in the molecule (33). Therefore, glycopeptide maps for both gp90 and gp45 were generated to determine whether the carbohydrate component of these two antigens also contributes to the structural and, perhaps, antigenic variation of EIAV. Structural changes in the glycopeptides could be detected in both gp90 and gp45 from these four virus strains. In fact, the available data suggest that at least two distinct patterns of glycosylation are possible for each glycoprotein molecule, and that the expressions of these glycosylation patterns in gp90 or gp45 from a single isolate are independent of one another. The observed presence or absence of entire families of glycopeptides in both gp90 and gp45 from different isolates may indicate the complete gain or loss of a glycosylation site. Such changes in glycosylation patterns are capable of introducing dramatic changes in antigenicity, as has been shown to occur in the hemagglutinin molecule from H5N2 avian influenza virus (20). The hemagglutinin from the virulent form of this virus possesses four amino acid sequence changes when compared with that of the avirulent form; one of these sequence changes completely abolishes a single glycosylation site, causing the subsequent complete loss of one of five mapped antigenic sites. In addition, recent studies on the nature of immune responses to Rauscher murine leukemia virus gp70 indicate that carbohydrate moieties not only can be major antigenic targets, but also can mask potential antigenic epitopes along the underlying polypeptide structure (1). Thus, the observed variation in glycosylation of the two EIAV surface antigens gp90 and gp45 may play a significant role in antigenicity of the virus during persistent infections.

Two-dimensional mapping of the RNA genomes of the four virus isolates revealed unique oligonucleotide profiles for each isolate. The genomic diversity observed during persistent infection by EIAV can be explained by several hypotheses. First, one could speculate that the EIAV inoculum contained a mixture of viruses and that the periodic disease episodes result from a sequential immune selection of a mixed population during infection. Although the data presented cannot definitively eliminate this possibility, there exists no precedent for a virus system in which minor variant populations are sequentially selected by immune responses. In fact, logic would argue that during the initial infection process, minor populations would be less likely than the predominant virus population to escape the host's immunosurveillance system and establish themselves in the animal. Thus, it is more probable that the variants are generated during persistent infection, as described for other retroviruses (8, 10, 48, 50, 53).

A second possible explanation for the variation in EIAV isolates is recombination, which has been observed with other retroviruses. However, in the absence of preexisting virus mixtures in the inoculum, recombination would require endogenous EIAV sequences. Unlike murine retroviruses, where large numbers of endogenous proviruses exist, endogenous EIAV-related sequences evidently do not occur in normal horse DNA or the DNA of other members of the genus *Equus* (45, 46; our unpublished observations). Moreover, recombinants between exogenous and endogenous retroviruses typically reveal large numbers of altered nucleotides when compared with the parental virus genomes by oligonucleotide mapping (9). Large numbers of altered oligonucleotides would also be expected if unique virus isolates resulted from genomic additions or deletions.

Thus, the level of genomic variability observed in EIAV is most compatible with the concept that during replication EIAV undergoes genomic point mutations at a relatively high frequency, some of which result in altered glycoprotein structures and antigenicity. This mechanism is further supported by the high rate of spontaneous mutations observed in avian and murine oncoviruses (10, 38, 50, 53), perhaps reflecting a lack of fidelity in reverse transcription in general.

Although the precise extent of genomic variation must await genomic sequencing studies, it is interesting to note that the total number of oligonucleotide alterations observed between the first virus isolate (P3-1) and the last virus isolate (P3-4) is no greater than the total number of alterations observed between any two consecutive isolates. Thus, there does not appear to be an accumulation of point mutations. Instead, it appears that the observed changes are due to the presence or absence of a specific, limited subset of oligonucleotides, whereas the majority of the oligonucleotides remain constant. Unique oligonucleotide patterns then appear to be obtained by various permissible combinations of this oligonucleotide subset. As shown in the localization studies involving P3-3, two oligonucleotides of this varying subset are apparently located in the *env* coding region of the RNA genome (Fig. 7). Thus, point mutations introduced in the *env* gene during the replication cycle of EIAV could translate into altered glycoprotein structure and altered antigenic properties.

It should be emphasized that the peptide and oligonucleotide mapping procedures employed here provide only a minimum estimate of total structural alterations. In the case of peptide mapping, only those peptides containing tyrosyl or histidyl residues (or both) susceptible to iodination are monitored; peptides lacking these residues, and perhaps undergoing variation, do not appear in the maps. Similarly, the 45 to 50 unique high-molecular-weight oligonucleotides examined for each of the four virus strains comprise only 10 to 15% of the total EIAV genome, assuming an average

oligonucleotide size of 20 to 25 nucleotides (8, 43). In addition, only oligonucleotide differences repeatedly observed in multiple experiments were indicated in the presented results. Thus, the actual level of glycoprotein and genomic variation between isolates is certainly more extensive than revealed by the analytical procedures employed here.

The fact that any changes in peptide or oligonucleotide structure were detected at all between virus isolates recovered only 4 to 8 weeks apart from the same animal indicates that EIAV is apparently a highly mutable virus. However, this property is not limited to EIAV. Indeed, antigenic and genomic variations during persistent infections were first reported for visna virus. Most recently, isolates of human T-cell leukemia virus III (acquired immune deficiency syndrome virus) have been shown to exhibit nucleic acid sequence variation only in the *env* gene region, as demonstrated by restriction mapping, heteroduplex analysis (17), and genomic sequencing (44, 46). EIAV, visna virus, and human T-cell leukemia virus III have all been tentatively placed in the lentivirus subfamily of retroviruses, based on morphological or serological data or genomic sequence homology (or a combination) (7, 13, 51). Thus, this strengthens the case for the recognition of the capacity for relatively rapid antigenic and structural variations during persistent infections of animals as a common characteristic of this subfamily. However, the reports of frequent spontaneous genetic variation in a variety of avian, murine, and bovine oncoviruses (5, 10, 19, 38, 50, 53) may indicate a characteristic property of retroviruses in general, whose biological relevance has been relatively overlooked.

The dynamic nature of EIAV infections and virus variation offers a unique model for studying the mechanisms and nature of antigenic variation in a persistent retrovirus infection where the process is clearly of biological importance. Thus, studies are underway to identify and characterize the epitopes of the virion glycoproteins and the range and chemical nature of their variation. This information should, hopefully, serve as a basis for designing effective vaccination procedures for this economically important virus disease.

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