

In Vivo Replicative Status and Envelope Heterogeneity of Equine Infectious Anemia Virus in an Inapparent Carrier

CAROL H. KIM† AND JAMES W. CASEY*

Department of Microbiology, Immunology, and Parasitology, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

Received 13 August 1993/Accepted 14 January 1994

The distribution and replicative status of equine infectious anemia virus (EIAV) DNA in the tissues of a well-characterized inapparent carrier horse were established by using the PCR technique. The EIAV *pol* region could be amplified in all of the tissues tested, including the cerebellum and periventricular tissue, at concentrations approximately 10^5 -fold less than in the same tissue from an acutely infected horse. Further analysis of the EIAV genome, with primer pairs diagnostic for sequential stages of reverse transcription, suggests that EIAV DNA in the brain, liver, and lymph nodes was incompletely synthesized. The products of reverse transcription were found to diminish progressively during first-strand synthesis, while products indicative of second-strand synthesis were observed only in kidney and spleen DNA samples. Sequences specific for different regions of the envelope could not be amplified from any of the tissues of the inapparent carrier, suggesting that the envelope is highly variable and may be subject to extensive drift. Together, the data suggest that low levels of EIAV replication persist without causing clinical disease in an inapparent carrier.

Equine infectious anemia virus (EIAV) is a member of the lentivirus subfamily of retroviruses and is etiologically associated with a variety of clinical symptoms which include anorexia, pyrexia, weight loss, and anemia. Variability in disease severity depends principally upon the viral strain and the general health of the animal. An EIAV infection can lead to an acute lethal episode, but more often self-resolves and results in a persistently infected animal. Clinically asymptomatic horses are considered persistently infected inapparent carriers, as demonstrated by their ability to transmit EIAV to a naive animal by whole-blood inoculation (3–5).

A hallmark of EIAV infection is the occurrence of febrile episodes which appear to be associated with variation in the envelope region of the virus. Increased viral replication is associated with these febrile spikes, along with recrudescence of the disease and clinical symptoms (11). One hypothesis for this cyclical recurrence and viral persistence is that as the immune system clears the predominant viral population during infection, distinct variants of EIAV which later emerge are selected (2, 6). These variants seem to have alterations confined to the envelope region of the virus (12). Over time, recurrence of febrile episodes and acute disease becomes less frequent and the animal eventually reaches a clinically asymptomatic state (4).

The replicative status of EIAV in inapparent carrier animals is not well understood (9). Because of the low levels of viral DNA in tissues of an inapparent carrier, detection of the viral genome by Southern blot procedures has not been possible (7, 14). As a first step to a better understanding of the mechanisms involved in EIAV suppression or latency, we examined selected regions of the viral DNA genome in different tissues of a well-characterized, clinically asymptomatic animal by using the PCR. The replicative status of EIAV in the tissues of this

inapparent carrier was elucidated by utilizing oligonucleotide primers that were diagnostic for progressive steps in reverse transcription (Fig. 3).

This inapparent carrier (horse 7) was maintained in the Cornell Equine Facility for 23 years after being experimentally infected with a Wyoming field isolate of EIAV (4, 5). An initial acute phase of infection was followed by three febrile episodes in the first year. Since that time, the animal displayed no clinical signs of EIA but remained antibody positive by the Coggins test. The acutely infected animal (horse 94) was experimentally infected with the highly virulent Wyoming strain of EIAV as previously described (7, 14). Tissues from the cerebellum, the periventricular region of the brain, the kidneys, the liver, the spleen, and the lymph nodes were removed from these horses and maintained at -80°C . DNA was prepared as previously described (14). The PCR and Southern blot analysis were carried out as previously described (7). The PCR primers and probes used in this study are identical to those used to investigate the status of EIAV DNA in an acutely infected horse (7) (Fig. 1).

Initially, the distribution of EIAV DNA in the tissues of the inapparent carrier horse was investigated by using PCR primers F and G, which span a region of the *pol* gene of the virus (Fig. 1). The samples analyzed included DNA from the cerebellum, the periventricular tissue of the brain, the kidneys, the liver, the spleen, and the lymph nodes and were compared to the positive control (100 pg of clone EIAV-12). When these samples were subjected to PCR amplification, a strong signal was visualized in all lanes. The autoradiograph of the PCR *pol* blot was probed with an internal ^{32}P -end-labeled oligonucleotide primer, I, which is internal to primers F and G (see Fig. 4).

Once it was established that EIAV DNA could be amplified from the DNA of all of the tissue samples tested, including the brain (by using the *pol* primers), the relative levels of the products were quantitated. Log dilutions of full-length lambda clone EIAV-12 (from 100 pg to 10 fg) and EIAV DNA from the acutely infected horse (1 μg to 100 pg) (8) were used as parallel standards to quantitate EIAV DNA from the inapparent carrier. Figure 2 shows that lanes 6 and 15 are approximately equal in intensity, indicating that the level of viral DNA

* Corresponding author. Mailing address: Department of Microbiology, Immunology, and Parasitology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853. Phone: (607) 253-3579. Fax: (607) 253-3419.

† Present address: Department of Microbiology, Oregon State University, Corvallis, OR 97331.

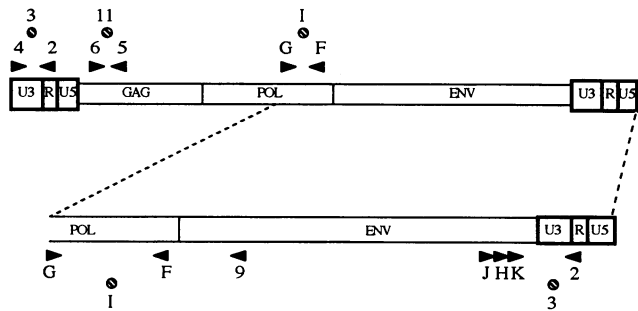


FIG. 1. EIAV genome diagram indicating the locations of the oligonucleotide primers and probes used for PCR experiments and Southern blotting. The arrowheads show primer polarity, and the circles represent the oligonucleotides used as end-labeled probes for hybridization analysis.

in the inapparent carrier liver is conservatively 10^5 times less than that in the same tissue of the acutely infected horse. Comparative quantitation of EIAV *pol* in the inapparent carrier and the acutely infected animal indicated that approximately 10^{-4} to 10^{-5} cells were infected in the former. This estimate is based on the level of EIAV DNA in the liver of the acutely infected animal, which approximates five copies per cell, assuming that all cells are potential targets for infection (14).

Additional PCR primers based on the sequence of a molecular clone isolated from the cell-adapted Wyoming strain of EIAV were used to characterize further the status of EIAV DNA in the inapparent carrier (15, 17). Primers 2 and 4 spanned a 183-bp region of the long terminal repeat (LTR), F and G spanned a 442-bp region of the *pol* gene, 5 and 6 spanned a 290-bp region of the *gag* gene, and 4 and 5 spanned the 5' LTR into the 5' portion of *gag* (Fig. 1). On the basis of the accepted model of reverse transcription, these primers were selected to be diagnostic for sequential stages of retroviral replication (Fig. 3).

Primers 2 and 4, spanning the LTR region from U3 to R, should amplify only those viral DNA replicative intermediates that have completed minus-strand strong-stop synthesis and the first jump. A predicted DNA fragment of 183 bp was detected in all tissues (Fig. 4). It should be emphasized that primers 2 and 4 did not distinguish between the 5' and 3' LTRs. However, amplification of this region could only have

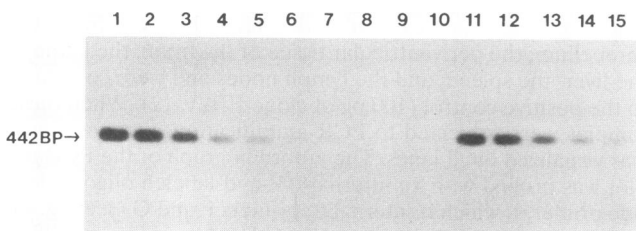


FIG. 2. PCR quantitation of EIAV DNA in the inapparent carrier horse. Primers F and G were used to amplify *pol* sequences from clone EIAV-12 (lanes 1 to 5), horse 7 liver DNA (lanes 6 to 10), and horse 94 liver DNA (lanes 11 to 15). The Southern blots were probed with internal oligonucleotide I. Lanes 1 to 5 contained log dilutions of EIAV-12, with lane 1 containing 100 pg of input DNA. Lanes 6 to 10 contained log dilutions of horse 7 liver DNA, with lane 6 containing 1 μ g of input DNA. Lanes 11 to 15 contained log dilutions of horse 94 liver DNA, with lane 11 containing 1 μ g of input DNA.

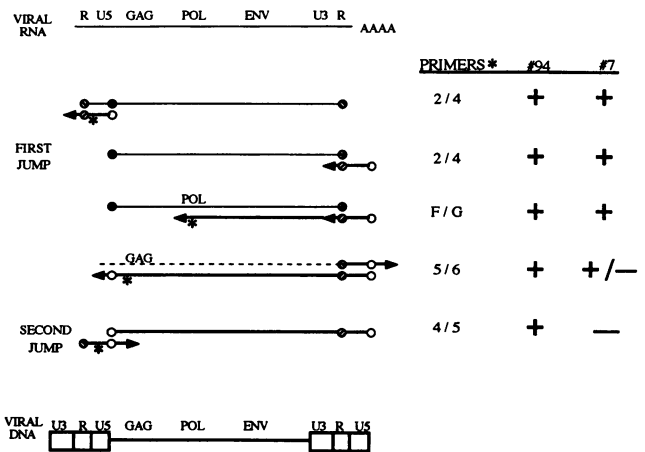


FIG. 3. The reverse transcription scheme of retroviruses. The sequential stages of reverse transcription are outlined, and the locations of PCR primer pairs are indicated by asterisks. A comparison of the stages in which reverse transcription was completed in acutely infected horse 94 and inapparent carrier horse 7 is displayed in tabular form at the right. A plus sign signifies amplification of EIAV DNA with PCR primers; a minus sign represents the inability of PCR primers to direct amplification of EIAV sequences. Data for horse 94 are summarized from reference 7.

been achieved if strong-stop synthesis and the first jump had occurred in these tissues. Primers F and G, specific for a segment of the *pol* region, have been discussed above and showed the presence of EIAV in all tissues of the inapparent carrier (Fig. 4). This observation also indicates that progressive synthesis of minus-strand DNA through *pol* was completed in these tissues. However, as elongation of minus-strand DNA progresses to *gag*, PCR amplification levels diminish in all of the tissues. This is evidenced by the dramatic decrease in amplification with primers 5 and 6, which span 290 bp of the *gag* region (Fig. 4).

Primers 4 and 5, which span the region between U3 and *gag*, were employed in the PCR to assay for second-strand synthesis (second jump). PCR amplification products indicative of this step were visualized only in spleen and kidney samples. Therefore, it appears that the potential for synthesis of EIAV would occur primarily in these tissues and that most tissues from the inapparent carrier horse contain incompletely synthesized viral genomes. These data are summarized in Fig. 3.

As different regions of the EIAV genome in the tissues of the clinically asymptomatic animal were examined, the PCR analysis with envelope region primers gave surprising results. No signal was observed in any of the tissues with primer pair H and 2, which amplifies a 585-bp fragment from the 3' end of *env* (Fig. 5). Next, primers J and 2, which span a 714-bp region starting farther upstream of H, were used for amplification. These primers were chosen in the event that the sequence of H in the envelope region of the clinically asymptomatic horse might differ from the published sequence. However, this PCR also failed to amplify the predicted EIAV envelope sequence (Fig. 5). An additional 3' envelope primer pair, K and 2, was employed, but again no amplification was achieved (Fig. 5). It should be noted that of the primers used as described above, primer 2 was shown to generate a product when paired with primer 4 to amplify LTR sequences (Fig. 4). Therefore, it is certain that at least one primer of the pair (primer 2) is capable of annealing to its target. Further, the hybridization probe, oligonucleotide 3, was shown to hybridize specifically with the

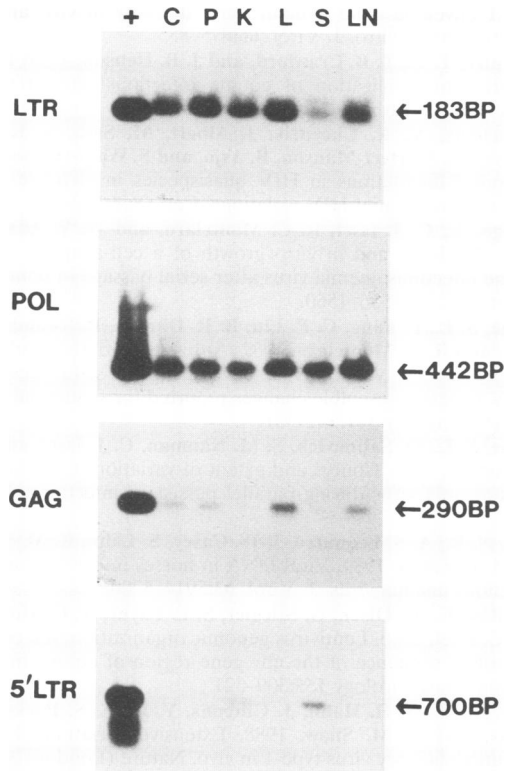


FIG. 4. Replicative status of EIAV DNA in the inapparent carrier horse. Primers 2 and 4 amplified LTR sequences, and primer 3 was the internal probe used for Southern blot analysis; primers 5 and 6 amplified the *gag* region of EIAV, and primer 11 was an internal hybridization probe; primers F and G amplified *pol* region sequences of EIAV, and primer I was an internal hybridization probe. One microgram of tissue DNA and 100 pg of EIAV-12 control DNA were used for these PCRs. The exposure time for the *pol* and LTR blots was 24 h, while that for the *gag* and 5' LTR blots was 72 h. Symbols: +, EIAV-12; C, cerebellum; P, periventricular tissue; K, kidney; L, liver; S, spleen; LN, lymph node.

LTR amplified product, showing that our inability to detect envelope amplification is not due to use of an inappropriate hybridization probe.

Oligonucleotide primers J, H, and K were all located at the 3' end of the *env* gene. To analyze the *env* region more fully, primers G and 9, spanning 1,141 bp from the 3' end of *pol* to the 5' end of *env*, were employed. Similarly, no amplification of EIAV DNA from the tissue of the inapparent carrier horse was observed with these upstream primers. It should be noted that primer G was used successfully to amplify a *pol* product in the previous experiments (Fig. 2) and oligonucleotide I was shown to hybridize specifically to this product. To increase the sensitivity of *env* detection, primary amplification with primers J and 2 was carried out for 35 cycles and followed by a secondary 35-cycle amplification with nested primers H and 3. Despite this additional amplification, an EIAV envelope product was not observed (data not shown).

At the DNA level, EIAV envelope sequences could not be amplified, even with nested PCR. At this point, we considered two possibilities: (i) these viral replicative intermediates had deleted envelope sequences, or (ii) the nucleic acid sequence of the envelope gene had diverged from the prototype sequence (15) to such an extent that the oligonucleotide primers chosen could no longer anneal to the target gene or serve as a

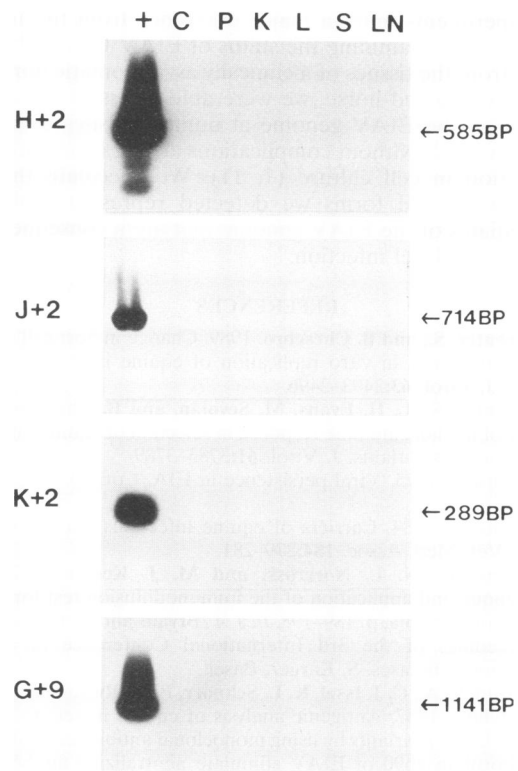


FIG. 5. PCR analysis of envelope regions in the inapparent carrier horse. Primers H and 2, J and 2, K and 2, and G and 9 were used to amplify envelope regions of EIAV DNA. These Southern blots were probed with oligonucleotide 3 for the first three primer pairs and with oligonucleotide I for G and 9. One microgram of tissue DNA and 100 pg of EIAV-12 control DNA were used for these PCRs. The blots were exposed for 72 h.

primer for polymerization because of extensive mismatch at the 3' end of the primer. We favor the second option, as there is much evidence for antigenic variation and genomic heterogeneity in EIAV (6, 7, 11-13) and its well-characterized relative, human immunodeficiency virus (HIV) (8, 10, 16, 18). In the HIV system, the concept of quasispecies resulted from the observation of extensive genomic complexity within the infecting viral population (10). These studies confirmed the enormous viral complexity that exists in vivo. In the EIAV system, isolates derived from sequential febrile episodes have been shown to display minor differences in envelope sequences (11-13). In addition, we have detected this diversity in an acutely infected horse in which EIAV subspecies were segregating to specific tissues on the basis of their envelope-specific PCR amplification products (7).

We interpret the PCR results to indicate that the replicative cycle of EIAV was complete only in the spleen and kidney tissues of the inapparent carrier horse. The tissues of the cerebellum, the periventricular region of the brain, the liver, and the lymph nodes contained incomplete viral genomes that had achieved the first jump but failed to complete second-strand synthesis. In an analogous study conducted by Zack et al. (18), quiescent T cells infected in vitro were found to contain incomplete HIV genomes. It was hypothesized that these incomplete viral genomes were labile intermediates of the HIV latent state and that subsequent stimulation may allow for virus production. However, the in vivo aspects of our

PCR experiments mark a major difference from the in vitro HIV study. By examining the status of EIAV DNAs obtained directly from the tissues of a clinically asymptomatic horse and an acutely infected horse, we were able to assess the in vivo structure of the EIAV genome at numerous sites within the infected animals without complications arising from additional propagation in cell culture (1, 11). We speculate that the predominant viral forms we detected represent replicative intermediates of the EIAV genome that are a consequence of continual low-level infection.

REFERENCES

1. **Carpenter, S., and B. Chesebro.** 1989. Change in host cell tropism associated with in vitro replication of equine infectious anemia virus. *J. Virol.* **63**:2492-2496.
2. **Carpenter, S., L. H. Evans, M. Sevoian, and B. Chesebro.** 1987. Role of the host immune response in selection of equine infectious anemia virus variants. *J. Virol.* **61**:3783-3789.
3. **Coggins, L.** 1975. Viral persistence in EIA. *Cornell Vet.* **73**:143-151.
4. **Coggins, L.** 1984. Carriers of equine infectious anemia virus. *J. Am. Vet. Med. Assoc.* **184**:279-281.
5. **Coggins, L., N. L. Norcross, and M. J. Kemen.** 1973. The technique and application of the immunodiffusion test for equine infectious anemia, p. 189-198. *In* J. T. Bryans and H. Gerber (ed.), *Proceedings of the 3rd International Conference on Equine Infectious Diseases*. S. Karger, Basel.
6. **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.
7. **Kim, C. H., and J. W. Casey.** 1992. Genomic variation and segregation of equine infectious anemia virus during acute infection. *J. Virol.* **66**:3879-3882.
8. **Kusumi, K., B. Conway, S. Cunningham, A. Berson, C. Evans, A. K. N. Iversen, D. Colvin, M. V. Gallo, S. Coutre, E. G. Shpaer, D. V. Faulkner, A. deRonde, S. Volkman, C. Williams, M. S. Hirsch, and J. I. Mullins.** 1992. Human immunodeficiency virus type 1 envelope gene structure and diversity in vivo and after cocultivation in vitro. *J. Virol.* **66**:875-885.
9. **McGuire, T. C., T. B. Crawford, and J. B. Henson.** 1971. Immunofluorescent localization of equine infectious anemia virus in tissue. *Am. J. Pathol.* **62**:283-292.
10. **Meyerhans, A., R. Cheyneir, J. Albert, M. Seth, S. Kwok, J. Sninsky, L. Morfect-Manson, B. Asjo, and S. Wain-Hobson.** 1989. Temporal fluctuations in HIV quasispecies in vivo are not reflected by sequential HIV isolations. *Cell* **58**:901-910.
11. **Orrego, A., C. J. Issel, R. C. Montelaro, and W. V. Adams, Jr.** 1982. Virulence and in vitro growth of a cell-adapted strain of equine infectious anemia virus after serial passage in ponies. *Am. J. Vet. Res.* **43**:1556-1560.
12. **Payne, S. L., F. Fang, C. P. Liu, B. R. Dhruva, P. Rwambo, C. J. Issel, and R. C. Montelaro.** 1987. Antigenic variation and lentivirus persistence: variations in envelope gene sequences during EIAV infection resemble changes reported for sequential isolates of HIV. *Virology* **161**:321-331.
13. **Payne, S. L., O. Salinovich, S. M. Nauman, C. J. Issel, and R. C. Montelaro.** 1987. Course and extent of variation of equine infectious anemia virus during parallel persistent infections. *J. Virol.* **61**:1266-1270.
14. **Rice, N. R., A. S. Lequarre, J. W. Casey, S. Lahn, R. M. Stevens, and J. Edwards.** 1989. Viral DNA in horses infected with equine infectious anemia virus. *J. Virol.* **63**:5914-5200.
15. **Rushlow, K., K. Olsen, G. Stiegler, S. L. Payne, R. C. Montelaro, and C. Issel.** 1986. Lentivirus genomic organization: the complete nucleotide sequence of the env gene region of equine infectious anemia virus. *Virology* **155**:309-321.
16. **Saag, M. S., B. H. Hahn, J. Gibbons, Y. Li, E. S. Parks, W. P. Parks, and G. M. Shaw.** 1988. Extensive variation of human immunodeficiency virus type-1 in vivo. *Nature (London)* **334**:440-444.
17. **Stephens, R. M., J. W. Casey, and N. R. Rice.** 1986. Equine infectious anemia virus gag and pol genes: relatedness to visna and to the AIDS virus. *Science* **231**:589-594.
18. **Zack, J., S. J. Arrigo, S. R. Weitsman, A. S. Go, A. Haislip, and I. S. Y. Chen.** 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell* **61**:213-222.