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

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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 naïve 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 controls (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 32P-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...
in the inapparent carrier horse 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 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 these 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 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).

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 for 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.

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