Characterization of infectious molecular clones of equine infectious anaemia virus

S. L. Payne,1* J. Rausch,1 K. Rushlow,2† R. C. Montelaro,2 C. Issel,3 M. Flaherty,4 S. Perry,4 D. Sellon4 and F. Fuller4

1 Department of Molecular Biology and Microbiology, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, Ohio 44106-4960, 2 Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, 3 Department of Veterinary Science, Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky 40546 and 4 Department of Microbiology, Pathology & Parasitology, North Carolina State University, Box 8401, College of Veterinary Medicine, Raleigh, North Carolina 27606, U.S.A.

We have recovered five infectious molecular clones of the lentivirus equine infectious anaemia virus (EIAV). The clones were recovered from fetal equine kidney (FEK) cells infected with a virulent, cell culture-adapted virus stock (designated PV) and have been characterized at a molecular level. Each clone has unique envelope and long terminal repeat (LTR) sequences. We further investigated LTR sequence variation in the PV stock using PCR amplification to obtain additional LTR clones from infected FEK cells and from peripheral blood mononuclear cells (PBMCs) from animals experimentally infected with PV. Sequence analysis of resulting clones indicates a selection for different LTR populations in pony PBMCs compared to FEK cells. Finally, we observed that the cloned EIAV proviruses did not remain infectious when maintained in a derivative of pBR322. However, two proviruses have been stably maintained in a low copy number vector (pLG338-SPORT).

Animal lentiviruses cause a variety of disease syndromes and serve as important model systems for understanding complex host–virus interactions that may aid in our understanding of human immunodeficiency virus type 1-induced human disease. Equine infectious anaemia (EIA) virus (EIAV) causes a disease characterized by high titre plasma viraemia, fever and thrombocytopenia (Clabough et al., 1991; Issel & Coggins, 1979). Following the initial acute febrile episode which usually occurs within the first month after infection, a horse may develop chronic EIA which is characterized by recurrent clinical episodes. The rapid onset of disease, as well as the ability to recover virus from discrete febrile episodes make EIAV a unique system in which to analyse the mechanisms of lentivirus virulence and persistence.

Virulent molecular clones of EIAV are required to identify molecular determinants of pathogenesis. However, the commonly used cell culture-adapted EIAV strains are avirulent whereas field isolates of EIAV cannot be routinely propagated in cell culture (Carpenter & Chesebro, 1989; Kono et al., 1970). PCR can be used to obtain clones directly from the tissues of infected animals (Perry et al., 1992) but owing to cell type-restricted virus replication it is difficult to assess the ability of such clones to replicate or to obtain sufficient amounts of virus for in vivo experiments. However avirulent cell-culture adapted strains of EIAV have been back-passaged in horses to obtain virus stocks that grow in vitro while retaining the ability to cause disease (Orrego et al., 1982). In this study we used a well characterized, cell-culture adapted, virulent virus stock of EIAV designated PV (Issel et al., 1992; Rwambo et al., 1990) in an attempt to obtain molecular clones of EIAV that could be readily grown and manipulated in cell culture and had retained the potential to cause disease upon experimental infection of Shetland ponies. Such clones would provide the means to identify, at a molecular level, determinants of virulence.

The cloning strategy shown in Fig. 1(a) was designed to maximize the recovery of full-length proviruses from PV EIAV-infected fetal equine kidney (FEK) cells which contain both integrated and unintegrated proviral...
Fig. 1. (a) Cloning strategy used to recover infectious EIAV proviruses. The proviruses were originally recovered as permuted molecules containing a single LTR. (b) Strategy used to rearrange clones 19 and 44 as two LTR-containing proviruses in pLG338-SPORT.

Table 1. Stability of EIAV proviral clones maintained in pBR322

<table>
<thead>
<tr>
<th>Lambda clone designation</th>
<th>Virus production from DNA pool*</th>
<th>No. positive/ no. tested†</th>
</tr>
</thead>
<tbody>
<tr>
<td>05</td>
<td>Yes</td>
<td>1/2</td>
</tr>
<tr>
<td>15</td>
<td>Yes</td>
<td>1/2</td>
</tr>
<tr>
<td>18</td>
<td>Yes</td>
<td>0/3</td>
</tr>
<tr>
<td>19</td>
<td>Yes</td>
<td>1/3</td>
</tr>
<tr>
<td>44</td>
<td>Yes</td>
<td>6/6</td>
</tr>
<tr>
<td>65</td>
<td>Yes</td>
<td>2/3</td>
</tr>
</tbody>
</table>

* The production of infectious virus was determined by the production of cell-free RT activity. Supernatants from transfected feline (FEA) cells were passaged onto fresh uninfected FEA cells to confirm the production of infectious particles.
† The fraction of individual DNA preparations from each pool that yielded infectious virus.

DNA (Rasty et al., 1990). Cellular DNA was digested with MluI, which cuts within the long terminal repeat (LTR), to convert all proviral DNA to 8 kb linear molecules. This fraction was gel-purified and ligated to produce circular molecules containing a single LTR. The ligation mixture was then digested with SacI which cuts at a unique site in the EIAV provirus and ligated to lambda Zap (Stratagene) DNA. Approximately $7 \times 10^7$ phage were screened using $^{32}$P-labelled DNA probes from the gag-pol and env regions of EIAV and 10 apparently full-length proviruses were recovered and subcloned into a modified pBR322 vector (designated pBR322sac) which was prepared by insertion of a SacI linker into the EcoRV site of pBR322. For initial screening, provirus-containing plasmids were pooled, SacI-digested, ligated at low concentration, and used to transfect a feline cell line (FEA) that is permissive for EIAV replication (Benton et al., 1981). Individual DNA samples from each positive pool were then tested to identify infectious clones. Virus growth was monitored by assay of culture supernatants for reverse transcriptase (RT) activity (Threadgill et al., 1993) and SDS-PAGE and immunoblot analysis were used to confirm the production of EIAV (data not shown).

DNA pools representing six lambda clones yielded infectious virus but, as indicated in Table 1, subsequent individual plasmid preparations were frequently negative for virus production indicating that the proviral clones were not stable in pBR322sac. In the course of previous studies we observed that certain EIAV sequences are genetically unstable when propagated using standard high copy number plasmids in *Escherichia coli*. It was subsequently demonstrated that an A/T-rich region upstream of EIAV env can function as a strong promoter in *E. coli* (Cunningham et al., 1993) suggesting that provirus instability results from inadvertent expression of the envelope glycoproteins. Gregory et al. (1990) have reported that the expression of deleterious sequences from promoter-like sequences can be reduced by using low copy number plasmid vectors. Thus two infectious EIAV proviruses were subcloned into a derivative of pLG338 (Cunningham et al., 1993) as illustrated in Fig. 1(b). These clones (designated pSPEIAV19 and pSPEIAV44) are genetically stable and are routinely propagated in our laboratories without loss of infectivity.

Virus stocks were prepared from clones pSPEIAV19 and -44 and pBR322sac65 and replication was quantified by RT assays. As shown in Table 2 each virus replicated...
Table 2. Growth properties of virus derived from EIAV molecular clones

<table>
<thead>
<tr>
<th>Clone designation</th>
<th>RT activity (c.p.m./ml)*</th>
<th>FEK cells†</th>
<th>Primary equine macrophages‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSPEIAV19</td>
<td>7 × 10⁶</td>
<td>1 × 10⁵§</td>
<td></td>
</tr>
<tr>
<td>pSPEIAV44</td>
<td>9 × 10⁶</td>
<td>1 × 10⁵§</td>
<td></td>
</tr>
<tr>
<td>pBR322Sac65</td>
<td>2 × 10⁶</td>
<td>1 × 10⁵§</td>
<td></td>
</tr>
</tbody>
</table>

* RT activity in cell-free supernatants was determined as described previously (Threadgill et al., 1993).
† Virus supernatants collected from > 10⁶ FEK cells at 13 days p.i.
‡ Virus supernatants collected from 10⁵ equine macrophages at 9 days p.i.
§ Cytopathic effects observed by 9 days p.i.

To high titre in FEK cells and primary equine macrophages. To determine whether the virus derived from the infectious molecular proviruses retained the virulence of the parent PV virus, stocks derived from pSPEIAV19 and -44 were used to infect Shetland ponies. These two clones were initially chosen because they represent distinct EIAV isolates, based on restriction endonuclease mapping. Two animals were inoculated with cell-free supernatants containing greater than 1 × 10⁶ units of RT activity, and were monitored for elevated temperature and decreased platelet counts as signs of clinical disease. Virus replication was monitored by assay of serum RT levels, PCR amplification of peripheral blood mononuclear cell (PBMC) DNA, and by seroconversion as described previously (Whetter et al., 1990). Virus from each clone replicated in vivo as indicated by positive PCR signals at 8 and 5 days post-infection (p.i.) and positive serum RT assays at 8 and 9 days p.i., respectively. Both animals seroconverted by 45 days p.i. No clinical signs of disease were observed, with both temperature and platelet counts remaining normal in both animals for 60 days p.i.

Results obtained from the experimental infections described above prompted us to characterize each proviral clone in order to identify and define unique clones for additional studies. We sequenced the SU region of env using the dideoxynucleotide method with EIAV-specific oligonucleotide primers. Overall, the PV SU sequences are very similar to those previously reported for avirulent cell-adapted EIAV (Rushlow, 1986). As shown in Fig. 2, each clone has a unique SU sequence and four of five clones differ in a region previously identified as the principal neutralization domain of SU (Ball et al., 1992). This result may reflect the method used to derive the PV stock of EIAV, as PV is a neutralization escape mutant derived by 13 passages of virus in cell culture in the presence of a broadly neutralizing serum obtained from an experimentally infected pony at 203 days p.i. (Rwambo et al., 1990).

In light of previous reports of variation in the U3 region of the EIAV LTR (Carpenter et al., 1991; Payne et al., 1987) we also sequenced the LTRs of all proviral clones (Fig. 3). Among nine LTR sequences derived from lambda clones, five unique LTRs were identified. To determine whether additional LTR sequences were present in the PV stock, we used PCR amplification of PV-infected FEK cells to recover additional LTR clones. Among eight clones derived from FEK cells using PCR we found one additional U3 sequence (designated pW16).

Fig. 2. The deduced amino acid sequences of the surface glycoproteins of five infectious PV clones. PND, Principal neutralizing domain (as defined by Ball et al., 1992). The underlined amino acids indicate two potential N-linked glycosylation sites that undergo variation among this panel of virus isolates.
To extend these observations, we also investigated which LTR sequences in the PV stock predominate in vivo where EIAV replicates primarily in tissue macrophages (Sellon et al., 1992). We reasoned that this information might suggest which of the PV clones were the best candidates for further studies. Using PBMCs from two PV-infected ponies as a source of DNA we PCR-amplified, cloned and sequenced 13 LTR segments. Interestingly, none of these LTRs were identical to any of the clones derived from infected FEK cells. Instead, 10 of the pony-derived LTRs were similar to LTRs obtained directly from an animal infected with the highly virulent Wyoming (Wyo) strain of EIAV. We have previously reported a similar LTR variation/selection phenomenon among virus isolates recovered from an experimentally infected animal (Payne et al., 1987), but in those studies the LTR sequences in the starting inoculum were not well characterized and virus was subjected to a single round of replication in FEK cells prior to cloning. However, these data in combination with the data presented here suggest the in vivo selection of a different LTR population than that seen when virus is propagated in FEK cells. It should be noted that the LTR variants observed in the PV-infected animals may have arisen either by mutation and selection events or by the selection of a minor pre-existing population not detected in the PV stock.

Perry et al. (1992) have reported that the major sequence differences between virulent Wyo EIAV and avirulent cell culture-adapted virus are located in SU and the LTR. They constructed chimeric viruses containing the Wyo env gene and demonstrated that the restriction for Wyo replication in equine dermal (ED) cells is not imparted by SU sequences. These results support the hypothesis that Wyo LTRs contain cis-acting elements that contribute to cell type-restricted replication in FEK and ED cells. Thus we hypothesize that the LTR sequences representative of those recovered directly from tissues or PBMCs are selected against upon adaptation to growth in FEK or ED cells but are favoured upon re-adaptation to replication in animals. As we have shown that EIAV clones 19, 44 and 65 replicate to high titre in macrophages, the selection for a different LTR population after replication of the PV stock in animals may indicate that subtle mechanisms are involved in the selection process. That LTR sequence variation is responsible for cell type restrictions and may alter retrovirus virulence has previously been shown in a number of other systems (Corboy et al., 1992; Paquette et al., 1990; Small et al., 1989).

Several transcription factor binding sites have recently been identified in the U3 region of the EIAV LTR (Carvalho & Derse, 1993a, b) and these are indicated in Fig. 3. They include PEA2 motifs, non-consensus AP-1 sites, and ets motifs. Comparison of the LTRs derived from PV-infected FEK cells to those obtained from PV- or Wyo-infected animals reveals differences in both the type and number of various transcription factor binding sites. For example, many FEK cell-derived LTRs contain two PEA2 and two AP-1 sites whereas 10 of 13 animal-derived LTRs lack both PEA2 sites and one AP-1 site. Instead one can identify an additional ets motif in these LTRs. Carvalho & Derse (1993b) have demonstrated that PU.1, a macrophage- and B cell-specific transcription factor of the ets family, binds to the consensus sequence AACXGGAAG where the underlined bases can be reversed but must be complementary. This consensus is found in the TATA box proximal ets motif in the EIAV LTRs recovered from cell culture but is absent from the Wyo virus LTR as well as from 10 of 13
clones recovered from PBMCs. Instead, as noted by the arrow in Fig. 3, the TATA box proximal ets motif found in the Wyo virus LTR contains a C to G transition and, thus, does not match the consensus PU.1 binding motif described by Carvalho & Derse (1993b). However, the presence of two additional ets motifs in the Wyo virus LTR suggests that PU.1 or other ets family proteins may regulate transcription through the additional sites. The types of sequence variation found in the EIAV LTR are likely to influence virus replication in different cell types. We are currently constructing proviral clones that differ only in their LTR sequences to test this hypothesis.

We wish to thank Dr David Derse for helpful discussions concerning this manuscript. This work was supported by NIH grants CA-50168 (S.L.P.) and CA-49296 (R.C.M.). The Harold and Leila Mather Charitable Foundation Research Career Support Program (S.L.P.), and the U.S. Department of Agriculture 9102321 (F.F.).

References


(Received 26 July 1993: Accepted 24 September 1993)