Long terminal repeat sequences of equine infectious anaemia virus are a major determinant of cell tropism

S. L. Payne,1 K. La Celle,2† X.-F. Pei,1 X.-M. Qi,2‡ H. Shao,2§ W. K. Steagall,2|| S. Perry3 and F. Fuller3

1 Biology Department, The University of Texas at Arlington, Box 19498, Arlington, TX 76019-0498, USA
2 Department of Molecular Biology and Microbiology, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, OH 44106-4960, USA
3 Department of Microbiology, Pathology & Parasitology, North Carolina State University, Box 8401, College of Veterinary Medicine, Raleigh, NC 27606, USA

The Wyoming strain of equine infectious anaemia virus (EIAV) is a highly virulent field strain that replicates to high titre in vitro only in primary equine monocyte-derived macrophages. In contrast, Wyoming-derived fibroblast-adapted EIAV strains (Malmquist virus) replicate in primary foetal equine kidney and equine dermis cells as well as in the cell lines FEA and Cf2Th. Wyoming and Malmquist viruses differ extensively both in long terminal repeat (LTR) and envelope region sequences. We have compared the promoter activities of the Wyoming LTR with those of LTRs derived from fibroblast-adapted viruses by examining their abilities to drive a luciferase reporter gene as well as by construction of infectious molecular clones differing only in LTR sequence. Our results indicate that LTR sequences are a major restriction for growth of the Wyoming strain of EIAV in fibroblasts.

The macrophage-tropic retrovirus equine infectious anaemia virus (EIAV) causes persistent infections of equids. Infected horses present with an acute febrile illness characterized by fever, thrombocytopenia and viraemia. The acute disease episode may be followed, weeks or months later, by additional febrile episodes (Montelaro et al., 1993). The Wyoming strain of EIAV is a highly virulent field strain that replicates efficiently in vitro only in primary equine monocyte-derived macrophages (eMDM). Wyoming virus was adapted to replication in equine dermis (ED) cells by Malmquist et al. (1973) and this virus and its derivatives are commonly exploited for biochemical and molecular studies. As the Malmquist virus is avirulent, its utility for in vivo studies is limited. To address this problem, Malmquist virus was back-passaged in animals, and virulent viruses that retained the capacity for replication in fibroblasts were recovered (Orrego et al., 1982; Rwambo et al., 1990).

Sequence comparisons of the genomes of the Wyoming strain and the Malmquist virus and its derivatives reveal sequence differences throughout, but the most strikingly divergent regions between the strains are the long terminal repeats (LTRs) and envelope (env) region (Payne et al., 1998; Perry et al., 1992). EIAV also accumulates env and LTR mutations during persistent infections in vivo (Payne et al., 1987; Leroux et al., 1997). As Wyoming EIAV and its cell-culture-adapted derivatives differ both in cell type restriction and virulence, it has not been possible, by sequence analysis alone, to predict the relationship of sequence variation to specific biological characteristics. Our laboratory has recovered infectious molecular clones of EIAV with in vitro replication properties similar to the Malmquist strain (Payne et al., 1994). These clones have a variety of LTR sequences, but all are distinct from the Wyoming LTR. We have also recently described molecular clones that encode virulent viruses, which were obtained by replacing the LTRs and env region of an avirulent molecular clone with Wyoming-derived sequences (Payne et al., 1998). We have used these molecular clones, and their LTRs, to examine the effects of LTR sequence on cell tropism of EIAV.

EIAV LTR sequence variation can be striking, but is usually limited to a portion of U3 that contains elements demonstrated to function as transcriptional enhancers. Variation in this region includes duplications and/or deletions as well as nucleotide substitutions (Carpenter et al., 1991; Derse et al., 1987; Maury et al., 1997; Payne et al., 1994). Many of the EIAV LTRs characterized to date are derived from fibroblast-adapted viruses and contain binding sites for a variety of well-
characterized transcription factors including PEA-2, ATF-1 and PU.1. Comparison with the Wyoming LTR reveals that Wyoming is somewhat simpler, in terms its assortment of transcription factor binding sites. The Wyoming virus LTR lacks a PEA-2 motif, has a single ATF-1 site and contains three ets motifs. To date, transcriptional activity of the Wyoming virus LTR has not been examined in cells permissive for fibroblast-adapted virus. In the current study we compare the promoter activity of the Wyoming LTR to those derived from fibroblast-adapted EIAVs, and demonstrate that the Wyoming LTR is a major determinant of cell tropism.

A consensus sequence for the Wyoming LTR was derived by comparison of sequences recovered from infected eMDM and multiple tissues from an infected horse and is shown in Fig. 1(A). The Wyoming LTR sequences were rather homogeneous, with only a few nucleotide differences (data not shown). The consensus derived in our laboratory is identical to a consensus sequence reported by Maury et al. (1997). A comparison of the U3 region of the Wyoming LTR with those of two fibroblast-adapted EIAVs is shown in Fig. 1(B). The 19 and 44 LTRs are derived from infectious molecular clones that produce virus that replicates in FEA, FEK and ED cells (Payne et al., 1994).

The transcriptional activities of the 19, 44 and Wyoming LTRs were tested using a luciferase reporter, constructed by placing complete LTRs into the promoterless vector pGL2-Basic (Promega) to generate pWyoluc, p19luc and p44luc. Transfections were performed using the calcium phosphate transfection system (Life Technologies). Luciferase constructs were used at 5 µg of DNA per well and an SV40 β-galactosidase plasmid was used (1 µg per well) to control transfection efficiency. To assay for transactivation by EIAV Tat protein, an SV40tat plasmid was added at 1 µg per well. The luciferase assays were sufficiently sensitive to detect the low basal activities of some EIAV LTRs and were linear over a wide range. FEA, Cf2Th and D17 cell lines were used in these studies as they are permissive for replication of some cell-culture-adapted EIAV strains, and are thus biologically relevant cells in which to test EIAV promoter activity.

The data from transfection assays are summarized in Table 1. The results represent averages from three or four independent experiments. For ease of comparison the basal activity of pWyoluc was arbitrarily set to 1 for each cell line. The most striking data are the differences in transcriptional activity between the Wyoming LTR and those representing fibroblast-adapted viruses. The levels of Wyoming basal activity are 10- to 20-fold less than the 19 or 44 LTRs, and in one case the basal activity of the Wyoming LTR is approximately 70-fold less (compare Wyoming to 19 in FEA cells). Although the basal activity of the Wyoming LTR is relatively
poor, it is transactivated by approximately 15- to 35-fold in the presence of EIAV Tat protein. However, the absolute transcription levels remain comparable with the basal activities of the 19 and 44 LTRs, and well below the transactivated levels of these LTRs.

There are a number of notable differences between the Wyoming and fibroblast-adapted LTRs. For the purposes of our studies we sought to determine if we could define specific sequence variations that might alter tropism for fibroblasts. Thus we looked for consistent differences between EIAV LTRs derived from fibroblast-adapted viruses versus the Wyoming consensus. One notable difference is that all fibroblast-adapted viruses examined in our laboratory (Payne et al., 1994, and unpublished), as well as those reported by others (Maury et al., 1997) have either a CTTCC ets motif proximal to the TATA element or a PEA-2 motif elsewhere in the LTR. In the majority of cases, fibroblast-adapted viruses have both. In contrast, the Wyoming LTR lacks both of these motifs. It has been demonstrated that GTTCC motifs are important for EIAV LTR transcription in equine macrophages (Maury, 1994) and more recently Maury et al. (1997) demonstrated that fibroblast nuclear extracts do not bind specifically to this sequence. Also, nuclear extracts from fibroblasts, but not macrophages, interact with the PEA-2 motif (Maury et al., 1997). Prompted by these data, we mutated the Wyoming LTR as shown in Fig. 1(B) to ascertain if minimal sequence differences might dramatically alter the transcriptional activity of this LTR in fibroblasts.

Table 1 shows that the Wyomut LTR (containing a GTTCC motif; see Fig. 1) is indeed a stronger promoter than the wild-type Wyoming LTR, demonstrating average increases in basal transcription from 5- to 13-fold. In the presence of Tat, the average transcription level of the Wyomut LTR increased 12- to 59-fold over the levels of the wild-type Wyoming LTR. The activity of the Wyomut LTR remains 2- to 5-fold lower than the fibroblast-adapted LTRs, but is significantly stronger than the wild-type promoter. In the converse experiments, the TATA proximal ets core motif of the 19 and 44 LTRs was changed to GTTCC. However, in the context of these fibroblast-adapted LTRs, which differ significantly from Wyoming, the single nucleotide change did not decrease in promoter activity. Rather, the single nucleotide change resulted in negligible changes in transcriptional activity.

Introduction of a PEA-2 motif into the Wyoming LTR also modestly increased transcription from the Wyoming LTR in FEA and D17 cells. It is therefore interesting to speculate that such minor nucleotide changes as those we have studied, might represent the first steps in ‘adaptation’ of a Wyoming-like LTR to replication in fibroblasts (or cell lineages other than monocytes/macrophages). In fact, Maury et al. (1997) have recently presented data indicating that the LTR sequences recovered from healthy carrier animals are distinct from the LTRs obtained from animals undergoing febrile episodes, and that only the former contain PEA-2 motifs.

The data presented above strongly suggested that the Wyoming LTR might be unable to drive virus expression in fibroblasts, which could therefore contribute to the restricted replication of Wyoming virus in these cells. To directly test this possibility, we replaced the LTRs from the infectious molecular clone pSPEia19 with those of a consensus Wyoming LTR to create the infectious clone p19/woLTRs. This clone was constructed using a combination of conventional cloning and PCR ‘sewing’ strategies such that the final construct contained a 5’ Wyoming LTR and a 3’ Wyoming consensus LTR, with all other viral sequences identical to
Given the complexity and variation of EIAV LTRs, we considered it important to determine LTR involvement in cell tropism, as it is not uncommon for investigators to passage virus from animals to fibroblasts and back. Our data suggest that while this might facilitate some studies, it should probably be avoided. The Wyoming LTR, and perhaps those of other field strains, are apparently not suited to growth in fibroblasts. If such virus stocks were fibroblast-passaged, strong selective pressures would instead favour minor virus populations or mutants with more favourable LTR sequences. Finally, while we have not carried out comparative transcriptional analyses of our LTRs in macrophages, virus replication data suggest that the 19 and 44 LTR sequences are not deleterious in this context, as viruses derived from clones pSPeiav19 and pSPeiav44 replicate to high titre and cause extensive cytopathic effects in eMDM (Payne et al., 1994). Thus we are left to speculate as to the selective pressures that might favour Wyoming virus type LTRs in vivo. Studies are currently under way in our laboratories to examine the role of LTR variation in EIAV pathogenesis.

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References


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