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The Surface Envelope Protein Gene Region of Equine Infectious Anemia Virus Is Not an Important Determinant of Tropism In Vitro

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Virulent, wild-type equine infectious anemia virus (EIAV) is restricted in one or more early steps in replication in equine skin fibroblast cells compared with cell culture-adapted virus, which is fully competent for replication in this cell type. We compared the sequences of wild-type EIAV and a full-length infectious proviral clone of the cell culture-adapted EIAV and found that the genomes were relatively well conserved with the exception of the envelope gene region, which showed extensive sequence differences. We therefore constructed several wild-type and cell culture-adapted virus chimeras to examine the role of the envelope gene in replication in different cell types in vitro. Unlike wild-type virus, which is restricted by an early event(s) for replication in equine dermis cells, the wild-type outer envelope gene chimeras are replication competent in this cell type. We conclude that even though there are extensive sequence differences between wild-type and cell culture-adapted viruses in the surface envelope gene region, this domain is not a determinant of the differing in vitro cell tropisms.

Equine infectious anemia virus (EIAV), a lentivirus (8, 16), persistently infects horses and causes a disease that is characterized by periodic episodes of fever, thrombocytopenia, and viremia (10, 20, 37). EIAV infection, unlike that of most lentiviruses, results in disease expression after only a short (1- to 4-week) incubation period (6, 7, 11, 12, 22, 30). This feature makes EIAV a very useful animal model system for studying lentivirus pathogenesis. The only other lentiviruses described that have a short incubation period similar to that of EIAV are some laboratory-derived strains of simian immunodeficiency virus (13). EIAV has been shown to replicate in equine adherent leukocyte cultures (which consist primarily of peripheral blood macrophages), but these cultures are difficult to maintain for any extended time. Malmquist et al. (27) adapted the Wyoming strain of EIAV (wild-type virus) to replicate in equine dermis cells. This adaptation resulted in a loss of virulence for this highly pathogenic virus strain (18), although some pathogenic properties can be regained by passage in vivo without a concomitant loss of the ability to replicate in equine dermis cells (5, 30). The wild-type virus is restricted in its ability to replicate in equine dermis cells (24, 25). We have sought to investigate the basis of this restriction, since this feature is an important limitation to our ability to study in vitro properties of the virulent wild-type EIAV. We have previously reported the isolation and characterization of an infectious proviral clone (CL22) of EIAV that replicates in in vitro cultures of equine dermis cells, equine adherent mononuclear cells, and a canine thymoma cell line (Cf2Th) (51). Virus derived from CL22 persistently infects horses but does not induce disease in these horses. The envelope proteins of retroviruses are of considerable interest because they (i) can determine the cell tropism of a virus through interaction with receptors on the cell surface, (ii) are responsible for membrane fusion activity required for virus penetration, and (iii) are important targets of neutralizing antibodies and cytotoxic T cells in the immune response to retrovirus infection (34, 39, 41).

We report here the observation that non-cell culture-adapted wild-type EIAV is unable to perform an early event(s) in EIAV replication (attachment, penetration, and/or reverse transcription) in equine dermis and Cf2Th cell cultures. We also present here the first report of sequence information for the wild-type Wyoming strain EIAV proviral DNA obtained by polymerase chain reaction (PCR) amplification from infected horses. A comparison of this sequence with that of the proviral clone of cell culture-adapted virus that is infectious in equine dermis cells and in vivo reveals a high degree of identity between the long terminal repeats (LTRs) and the *gag* and *pol* genes, but the envelope gene region differs substantially. We have infected equine dermis cells with virus derived from two chimeric clones that contain wild-type sequence replacements in either all or the C-terminal half of the surface envelope protein gene and have also tested virus for the ability to productively infect equine macrophages. These two chimeric clones replicate productively in both equine dermis cells and equine macrophages with the same relative efficiencies as the parental pER-derived virus. These results demonstrate that the surface envelope protein of EIAV is not an important determinant of the tropic properties of wild-type virus for replication in equine macrophages in vitro and that the complete restriction of wild-type virus replication in equine dermis cells is not determined by the wild-type surface envelope protein.

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MATERIALS AND METHODS

Virus and cells. The wild-type Wyoming virus strain 158 was obtained from L. O. Mott (20) and propagated in horses. Serum was obtained from an infected horse during the peak febrile response, and the titer of this stock (hereafter referred to as wild-type Wyoming) was determined to be approximately 10^6 infectious doses per ml (as determined by horse inoculation assay). The Malmquist strain of EIAV was derived from the wild-type Wyoming strain by adaptation of the wild-type virus by multiple blind passages in equine dermis fibroblast cells (27). Viruses derived from the molecular clone CL22 or the molecular clone pER were grown in equine dermis cells (ATCC CCL 57). Equine dermis cells were cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum. Canine fetal thymus cells, Cf2TH (ATCC CRL 1430), were cultured in Dulbecco modified Eagle medium with 5% fetal bovine serum.

Equine adherent macrophage cultures were established by Ficoll separation of blood cells and plating for 24 h, at which time nonadherent cells were removed by washing twice with RPMI plus 10% fresh horse serum as previously described (51).

Early events in EIAV infection. Wild-type Wyoming virus was concentrated from acutely infected horse serum (9 to 12 days postinfection) by ultracentrifugation (100,000 × *g* for 1 h) through a 20% sucrose layer. The concentrated virus was resuspended in phosphate-buffered saline (PBS) at 1/100 the original volume. This step was necessary to obtain a sufficient inoculum of virus to infect equine macrophage and equine dermis cell cultures and to more accurately determine reverse transcriptase activity, since we have found that equine serum interferes with this assay. Equine dermis cells or equine macrophages were infected with 0.2 ml of either pER-derived virus (see below) or a concentrated wild-type virus stock (each of which yielded about 16,000 cpm in reverse transcriptase assays). The virus was adsorbed for 2 h at 37°C either in the presence of 10 μg of Polybrene per ml (equine dermis cells) or without Polybrene (equine macrophage cultures). After 2 h the inoculum was removed and Dulbecco modified Eagle medium plus 10% fetal bovine serum was added. At 12 and 24 h postinfection the cells were removed by trypsinization, washed twice in 1× PBS, and lysed in a buffer (0.01 M Tris [pH 8.0], 0.1 M sodium chloride, 0.025 M EDTA) containing 0.5% sodium dodecyl sulfate and 100 μg of proteinase K per ml. Preparations were incubated overnight at 37°C and extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and the DNA was precipitated with ethanol. PCR amplification was performed as previously described (50) using the primer pair *envB*→ (5′-AATCAAATGCTATAGAATGCTG-3′) and ←*envB* (5′-GTATGATTTTGTACTTCCATTA-3′), which generates a 1.1-kb fragment. For growth experiments, cells were infected as described above and aliquots of supernatant were taken for reverse transcriptase assay at the indicated time points.

PCR amplification and cloning of wild-type virus. PCR amplification of mononuclear cell DNA from horses infected with the wild-type virus was carried out as previously described (51) except that the number of cycles was limited to 30 to maximize fidelity. Primer sequences spanning the genome were derived from the sequence of the proviral clone 1369 (21, 52). After amplification, the DNA product was isolated by agarose gel electrophoresis and subsequently purified with Geneclean from Bio 101 (La Jolla, Calif.). For blunt-end cloning, DNA was treated with polynucleotide kinase and ligated to *Sma*I-cut, dephosphorylated pGEM-4Z (Promega, Madison, Wis.). For other cloning experiments, DNA was digested with restriction enzymes and ligated to the appropriately prepared vector. Competent *Escherichia coli* DH5αF′ cells were transformed, and colonies were screened by hybridization with a ^{32}P-labelled probe prepared by nick translation of the insert DNA.

Sequence analysis of wild-type and CL22 proviral clones. Sequence determination of most of the wild-type clones was carried out on the double-stranded plasmid DNA essentially as described by Toneguzzo et al. (47), using Sequenase obtained from U.S. Biochemicals (Cleveland, Ohio). Other wild-type clones were sequenced by subcloning the inserted DNA into M13 and sequencing the single-stranded template with Sequenase. Plasmids containing EIAV proviral DNAs (clone 1369 and CL22) were sequenced as previously described (21).

Construction of replacement clones. (i) pER. The CL22 plasmid is extremely unstable when propagated in a number of *E. coli* strains, in contrast to clone 1369, a previously described noninfectious clone (21, 52). Among other possibilities, it was thought that the cellular flanking sequences of CL22 may contribute to its instability. Since CL22 and clone 1369 contain *Mlu*I sites 156 bp from their U3 termini, the 8.0-kb *Mlu*I fragment from CL22 was ligated to the *Mlu*I-digested plasmid background from clone 1369. This yielded an infectious molecular clone with a stable phenotype, designated pER.

(ii) pERrH3. The 735-bp *Hin*dIII restriction fragment (nucleotides [nt] 5775 to 6510) was removed from the wild-type clone pCD (see Fig. 3) and ligated into *Hin*dIII-digested, dephosphorylated pER(−)Hind (pER clone in which the *Hin*dIII fragment has been removed).

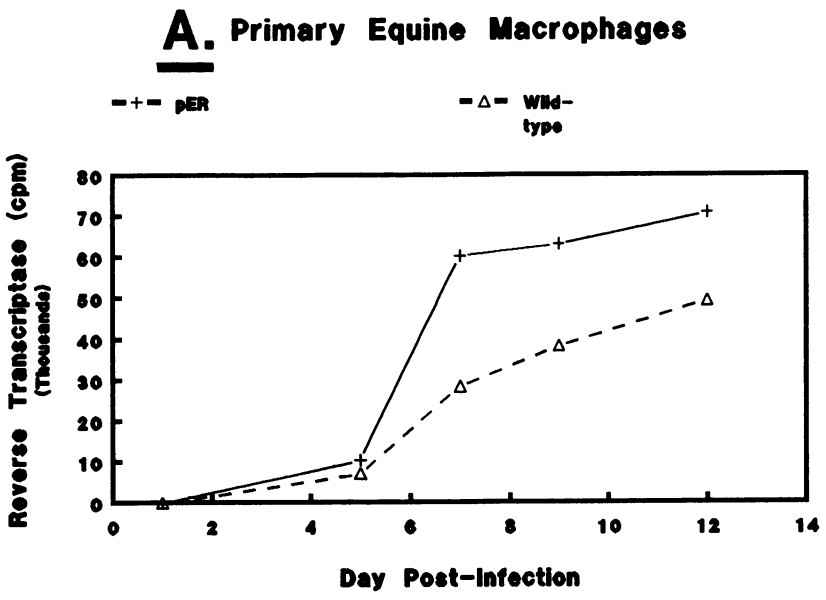
(iii) pERrNhe. To construct the replacement clone pERrNhe, the 1.5-kb *Bam*HI fragment from the wild-type clone pDB1-4 (see Fig. 3) was first inserted into the *Bam*HI site of pCD, resulting in a wild-type clone containing EIAV gene sequences from nt 3853 to 7872. The *Nhe*I fragment (nt 3910 to 6700) was then excised from this clone and ligated to *Nhe*I-digested, dephosphorylated pER(−)Nhe (pER clone in which the *Nhe*I fragment has been removed).

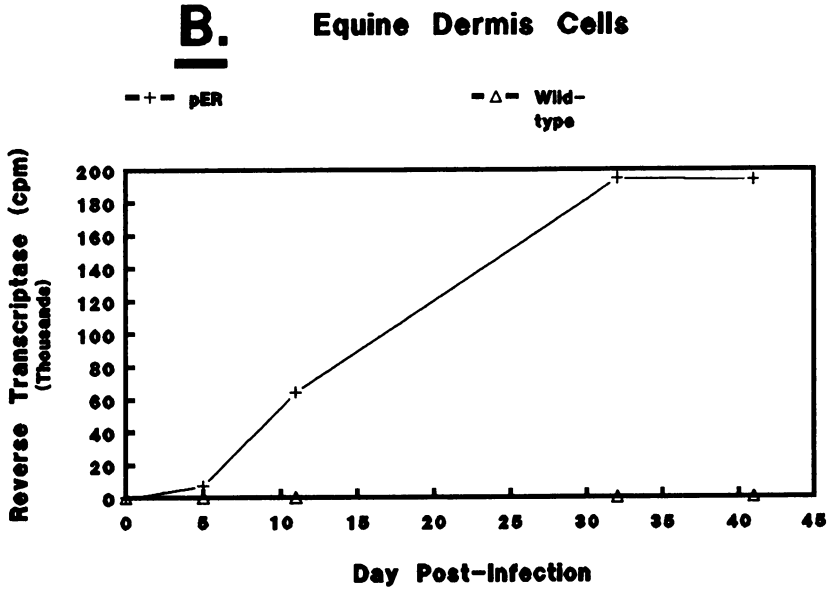
The structures of the wild-type replacement clones were confirmed by redigesting with restriction enzymes used in their construction and, additionally, sequencing through those sites to ensure that all junctions were intact.

Nucleotide sequence accession numbers. The sequences of CL22 and each of the individual PCR-derived wild-type Wyoming clones have been submitted to GenBank and have the following accession numbers: CL22, M87581; pU3Bm4, M87595; pU3Bm2, M87594; pApaBam, M87575; p523, M87574; pBgl, M87578, p518, M87572; p519, M87573; pKpnBgl, M87592; pKpn, M87591; pDB1-4, M87583; pCS3, M87582, pCD, M87580; pC39, M87579; pFD, M87587; pBBH-2, M87576; pBBH-3, M87577; pHU53, M87588; pHU531A, M87589; pHU531B, M87590; pDB4-50A, M87584; pDB4-50B, M87585; pDB4-50C, M87586; and pNsi-12, M87593.

RESULTS

Restriction of wild-type EIAV replication in equine dermis and Cf2Th cells. The wild-type Wyoming strain of EIAV that has never been cultivated in vitro is highly restricted in its ability to replicate in equine dermis and Cf2Th cells, as shown in Fig. 1B and C. The wild-type virus is able to infect and replicate in equine adherent macrophage cultures (Fig.





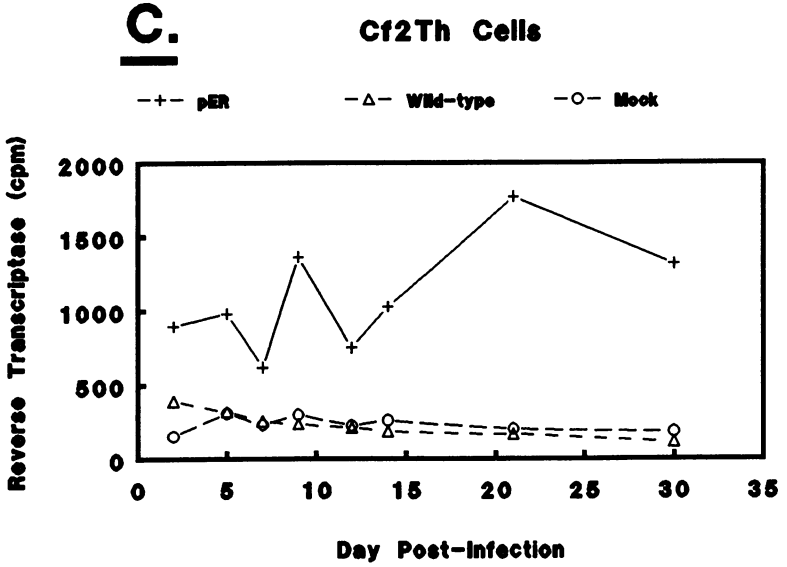


FIG. 1. Infection of primary equine macrophage, equine dermis, and Cf2Th cells with wild-type or pER-derived virus. Cell cultures were infected with a virus inoculum containing approximately 20,000 cpm of reverse transcriptase activity. At the times indicated postinfection, 10 μl of the culture supernatant fluid was assayed for reverse transcriptase activity. An EIAV-specific PCR assay performed on cell DNA from the last day of infection for each experiment indicated that while all cell types displayed PCR-positive signals for pER-derived-virus-infected cultures, only equine macrophage cultures were positive for wild-type virus (data not shown).

1A) but is unable to productively infect the equine dermis cells (Fig. 1B) or Cf2Th cells (Fig. 1C). In contrast, viruses derived from the molecular clones pER (Fig. 1) and CL22 (data not shown) are able to productively infect equine dermis cells, Cf2Th cells, and equine macrophages. The inability of wild-type virus to productively infect equine dermis cells continues for at least 3 months postinfection. The level of pER-derived virus replication as measured by reverse transcriptase activity (2) in Cf2Th cells was significantly lower than in equine dermis or equine macrophage cultures and is a consistent observation. Both the pER-derived virus and wild-type viruses appeared to be cytopathic in the equine adherent macrophage cultures.

Wild-type EIAV is unable to perform an early event(s) in virus replication in equine dermis and Cf2Th cells. We next sought to determine the basis of the restriction of wild-type virus in equine dermis and Cf2Th cells. In order to determine whether wild-type virus is capable of entering cells in vitro and reverse transcribing the viral genome, we infected equine dermis, Cf2Th, or equine macrophage cell cultures with either wild-type or pER-derived virus particles. Approximately 2×10^4 cpm of each virus, as determined by our reverse transcriptase assay, was used for the infections. At 12 and 24 h postinfection, the infected cells were lysed and DNA was extracted as described above. The protocol used for DNA extraction includes both integrated and unintegrated proviral DNA (our unpublished results). A PCR amplification was performed on a 1.1-kb portion of the envelope gene of EIAV, as shown in Fig. 2. Panel A shows the presence of pER-derived and wild-type viral sequences in macrophages. In contrast, we were unable to detect wild-type virus sequences in equine dermis cells (Fig. 2B) or Cf2Th cells (Fig. 2C), even though pER-derived virus sequences were readily amplified in these cells. We chose to use Polybrene during the attachment of virus particles to equine dermis and Cf2Th cells because we found about a 5- to 10-fold increase in the level of the PCR signal of pER-derived virus from these experiments (data not shown). In the absence of Polybrene we are also unable to detect wild-type viral DNA in equine dermis cells (data not shown) but could detect pER-derived viral DNA. No Polybrene was used for attachment of virus particles to equine macrophages because the Polybrene (even at 1 μg/ml) was highly toxic to these cell cultures. These results suggest that wild-type virus is incapable of performing an early event(s) in virus replication in equine dermis cells. This early event could involve (i) binding of the virus surface envelope protein to equine dermis cells due to the absence of cell surface receptors which are present on equine macrophages, (ii) entry of virus into cells, or (iii) synthesis of viral DNA by reverse transcriptase.

Comparison of wild-type non-cell culture-adapted Wyoming virus with an infectious proviral clone, CL22. We were interested in determining what differences might exist at the nucleotide sequence level between the cell culture-adapted, infectious molecular clone-derived virus (pER) and the non-cell culture-adapted, virulent Wyoming wild-type virus. The sequence of the infectious molecular clone CL22 (pER was derived from CL22; see Materials and Methods) was determined as previously described for the noninfectious EIAV proviral clone 1369 (21). Wild-type Wyoming virus sequences were determined as described in Materials and Methods by PCR amplification of mononuclear cell DNA from horses acutely infected with this virus and by subsequent cloning and sequencing. A schematic diagram showing the location of wild-type Wyoming virus clones derived by

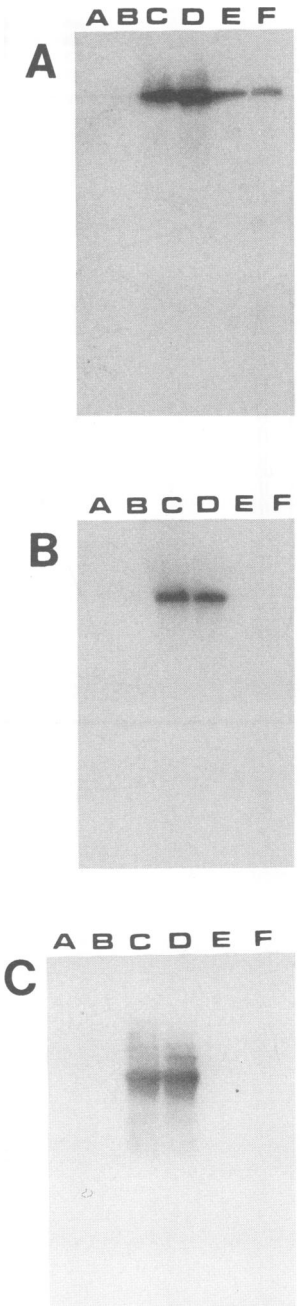


FIG. 2. PCR detection of input EIAV particle infection of equine macrophages (A), equine dermis cells (B), and Cf2Th cells (C). The cells were either infected with pER-derived virus (lanes C and D) or wild-type virus (lanes E and F) or mock infected (lanes A and B). After a 2-h attachment at 37°C the virus inoculum was removed, and at either 12 h postinfection (lanes A, C, and E) or 24 h postinfection (lanes B, D, and F) total cell DNA was extracted and samples were subjected to PCR amplification as described in Materials and Methods. The products of the PCR amplification were resolved on a 1% agarose gel, transferred to nitrocellulose, and subjected to Southern blot analysis with an EIAV-specific envelope gene probe radiolabeled by random priming.

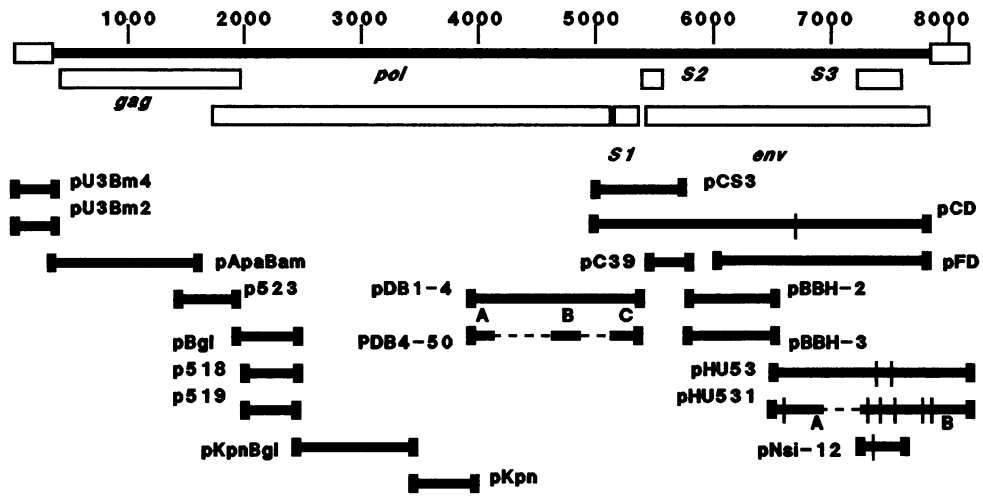


FIG. 3. Schematic diagram of the location of PCR-derived molecular clones of the wild-type Wyoming strain of EIAV. PCR amplification of peripheral blood mononuclear cell DNA from a wild-type Wyoming-virus-infected horse at 9 to 12 days postinfection was used to generate DNA clones spanning the entire genome of the wild-type provirus. All clones were completely sequenced except for the regions represented by the dashed lines. The small vertical lines indicate sites where in-frame termination codons were detected by our sequence analysis either in the envelope gene reading frame or in the S3 open reading frame.

PCR amplification of infected horse peripheral blood cells is shown in Fig. 3. Together these clones represent the full-length viral genome compared with previously reported EIAV proviral sequences (21, 38, 44). Thus, Fig. 4 shows that the alignment of the complete nucleotide sequence of CL22 with those of PCR-derived wild-type virus clones spans the entire CL22 genome. Note that the infectious provirus of CL22 is missing the first 35 nt of the U3 region in the 5′ LTR. We have summarized the sequence comparison between CL22 and the PCR-derived wild-type Wyoming clones in Table 1. The *gag* gene regions are highly conserved, with only a 0.26% nucleotide sequence difference and a 0.2% amino acid coding difference between them. In the *pol* gene region a difference of 0.67% in nucleotide sequence leads to a 0.78% amino acid coding difference. It is not surprising that the least-conserved regions between CL22 and wild-type sequences reside in the LTR (5.14% nucleotide difference) and the gp90^{env} surface protein gene region (5.57% nucleotide difference). In the LTR, the most dramatic difference between the wild-type sequence and that of CL22 is at positions 137 to 142, where a CAAT sequence motif in addition to the other CAAT sequence motif at positions 119 to 122 is present in the wild-type sequence. Our previous work has shown that cDNA clones derived from cell culture-adapted virus that had been passaged through ponies to restore virulence also have a double CAAT box motif (unpublished observations), suggesting a possible correlation with a virulent phenotype. The surface envelope protein, gp90, is the most variable between CL22 and the wild type (Fig. 5), with an amino acid coding difference of 11%, and the transmembrane protein shows a coding difference of 6.1%. One very interesting observation that we made in our analysis of two of the individual PCR-amplified clones was the clustering of in-frame termination codons in the gp45 gene region. The locations of these termination codons are shown schematically in Fig. 3 for the clones pCD, pHU53, pHU531, and pNSI-12. We found nine in-frame termination codons in gp45 or in the overlapping gene region S3 but only one in the C-terminal region of gp90 (Fig. 4). We did not observe in-frame termination codons in any other region of the viral genome. Two of these clones, pHU53, and pHU531A, and pHU531B, displayed a G→A hypermutation phenotype that is shown in Fig. 4. This hypermutation phenotype is similar to the observations for human immunodeficiency virus type 1 (HIV-1) described by Vartanian et al. (48).

It has been previously observed that processing of gp45 occurs by a cleavage C terminal to the membrane anchoring site of the transmembrane envelope protein to yield gp32 and p20 polypeptides (36). It has been suggested by Rice et al. (36) that the p20 protein found in virions may be unessential for viral infectivity (at least in vitro) (36), allowing some viral genomes to accumulate mutations in this region. Such mutants may have some survival advantage in a host that has developed an immune response, since accompanying mutations in the *rev*-like gene may favor a latent proviral state.

Significant differences were found in the open reading frames designated S1 and S2. The wild-type S1, which codes for the second exon of the *tat* protein (29, 45), was found to differ from CL22 by 3.09%, leading to a coding divergence of 2.19% (two coding differences). For S2, whose function is as yet unknown, amino acid differences were also noted at two positions.

The S3 (putative *rev* homolog) (42, 45) open reading frame displays 24 coding differences. Three clones that contained in-frame termination codons located within the region that shares significant homology with other lentivirus *rev* proteins were isolated. Two of these clones, HU53 and HU531, exhibited a G→A hypermutation phenotype described above, but one clone, Nsi-12, had a termination codon with no evidence of hypermutation. EIAV proviruses with a termination codon in the S3 open reading frame could not produce high levels of infectious virus if the putative EIAV *rev* protein functions in a fashion analogous to that of other lentivirus *rev* proteins. We would not have predicted that defective proviral templates would be so common in an acutely infected horse unless immune selection pressure favors the survival of *rev*-defective proviruses. Stephens et al. (45) have reported finding defective *rev*-like genes in cDNA clones isolated from Cf2Th cells unable to produce

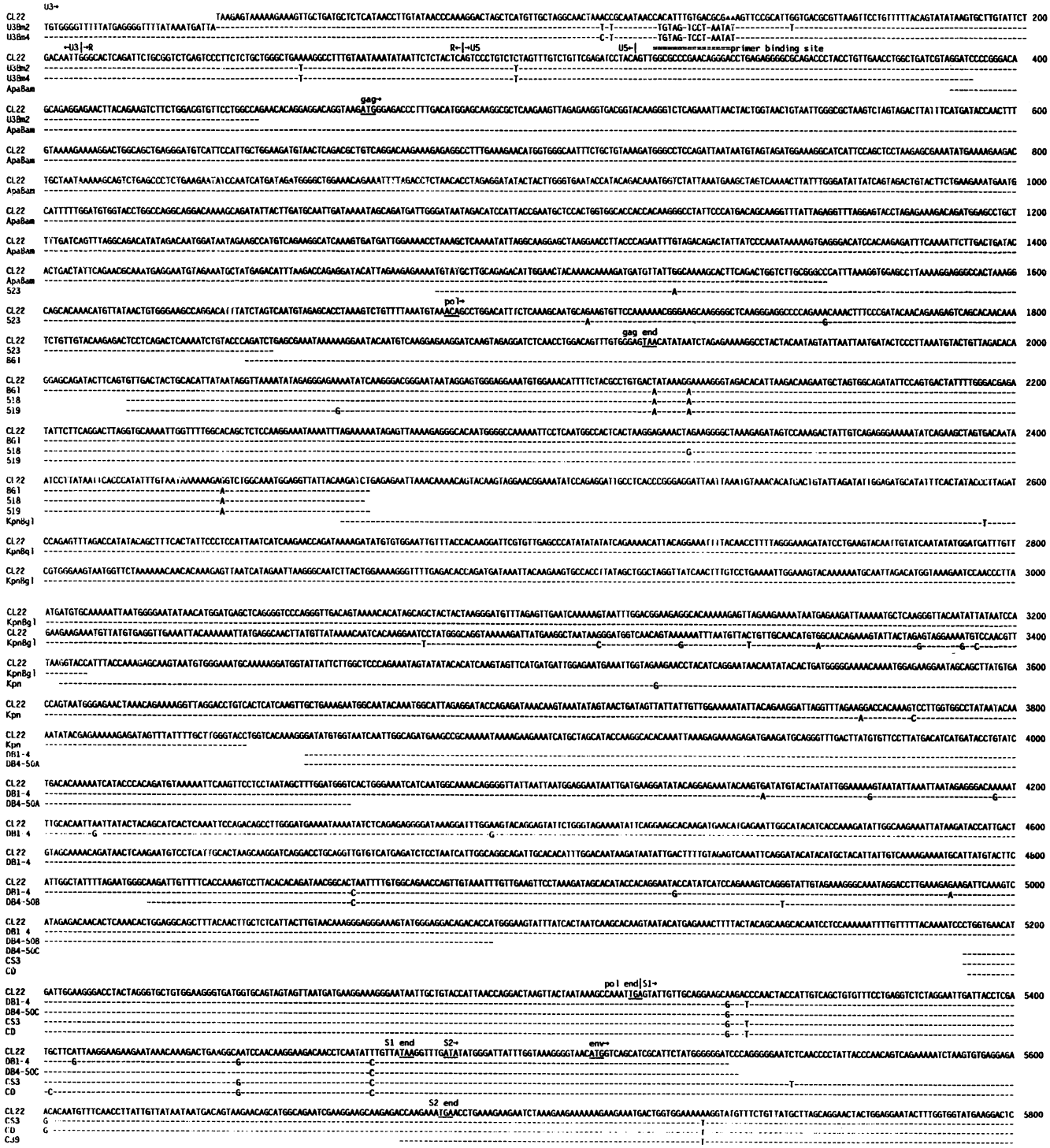


FIG. 4. Complete nucleotide sequence of the infectious molecular clone CL22 and the wild-type Wyoming (non-cell culture-adapted) virus. The nucleotide sequence was determined by supercoil sequencing as described in Materials and Methods. Dashes indicate identity with the CL22 nucleotide at that position, and solid triangles indicate a gap in the sequence at that position.

infectious virus, although in that case, *rev* expression was interrupted by a frameshift mutation.

The transmembrane envelope glycoprotein (gp45)-coding region of the wild-type virus did not contain as many coding differences as the gp90-coding region, with a 3% nucleotide difference resulting in a 6.1% amino acid coding difference. These results are consistent with those of a previous report demonstrating that gp45 is less variable than gp90 (9).

Phenotypic differences between viruses derived from wild-type surface protein gene chimeras and parental strains. In

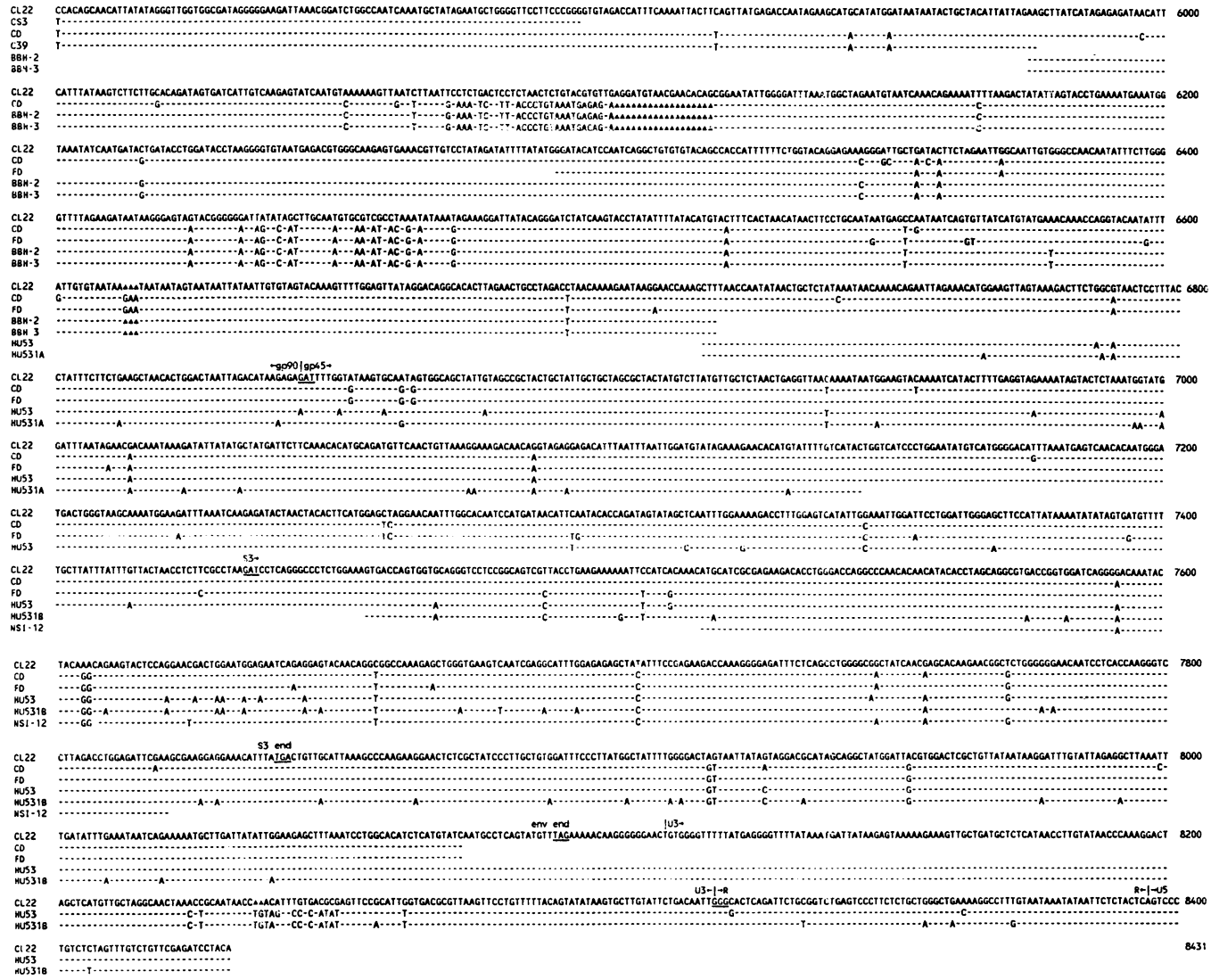


FIG. 4—*Continued*.

TABLE 1. Sequence comparisons of CL22 and wild-type Wyoming clones

Gene	Size (nt)	% Nucleotide difference	% Amino acid difference	No. of inframe terminal codons
5′ LTR	321	5.14	—[a]	—
gag	1,458	0.26	0.20	0
pol	3,441	0.67	0.78	0
S1	153	3.09	2.19	0
S2	198	0.58	1.74	0
env				
gp90	1,332	5.57	11.0	1
gp45	1,260	3.04	6.10	4
S3	408	3.82	8.16	5
3′ LTR	321	5.30	—	—

[a] —, noncoding.

order to examine whether the restriction of the wild-type virus in equine dermis cells is due to differences in the envelope gene which may lead to a loss of specific receptor recognition, we tested the effects of replacing the gp90-coding region of pER with wild-type sequences. The clone designated pERrNhe contains wild-type sequences from the *Nhe*I site at nt 3910 to a second *Nhe*I site at nt 6700 (Fig. 6). This sequence contains the carboxyl-terminal third of the *pol* gene coding region; all of S1, S2, and gp90; and the first 60 nt of gp45. A second chimeric clone, pERrH3, contains wild-type sequences from the *Hin*dIII site at nt 5775 to the *Hin*dIII site at nt 6510; thus, this clone contains wild-type sequences in only the gp90-coding region. Plasmid DNA, along with the neomycin drug resistance-bearing plasmid pSV5neo (5:1, provirus-pSV5neo) (43), was used to transfect Cf2Th cells by the calcium phosphate precipitation method (1). The Cf2Th cell line instead of the equine dermis cells was used for the transfection because it is not possible to grow the latter from single cells; therefore, drug selection cannot be used and transfection results in only a few infected cells in a population which is quickly overgrown by unin-

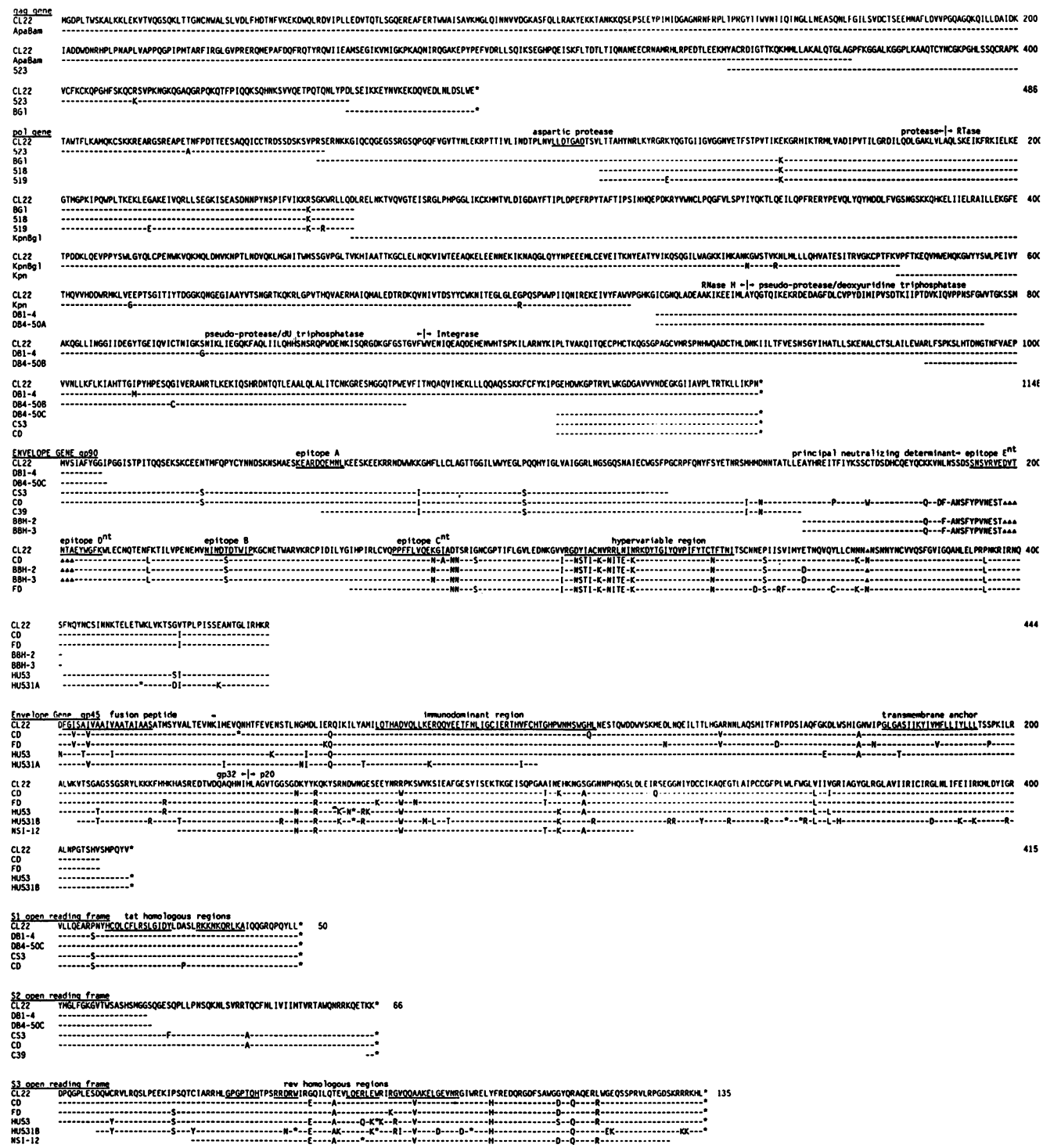


FIG. 5. Comparison of the predicted proteins of the avirulent cell culture-adapted CL22 virus and the wild-type Wyoming virus. The amino acid sequence is represented in single-letter code. Dashes indicate identity with the CL22 amino acid at that position, and solid triangles indicates a gap in the predicted sequence at that position.

fected cells (data not shown). After G418 drug selection, virus production was monitored by reverse transcriptase assay. Transfection of the Cf2Th cells results in a steady accumulation of virus, which then declines after approximately 4 to 5 weeks. The PCR signal remained strong, indicating that the provirus remained in the host cell DNA (data not shown). Virus was harvested from these cells, and cell-free supernatant was used to test the infectivity of virus from the chimeric clones in equine dermis, Cf2Th, and equine macrophage cell cultures. Figure 7 shows the results

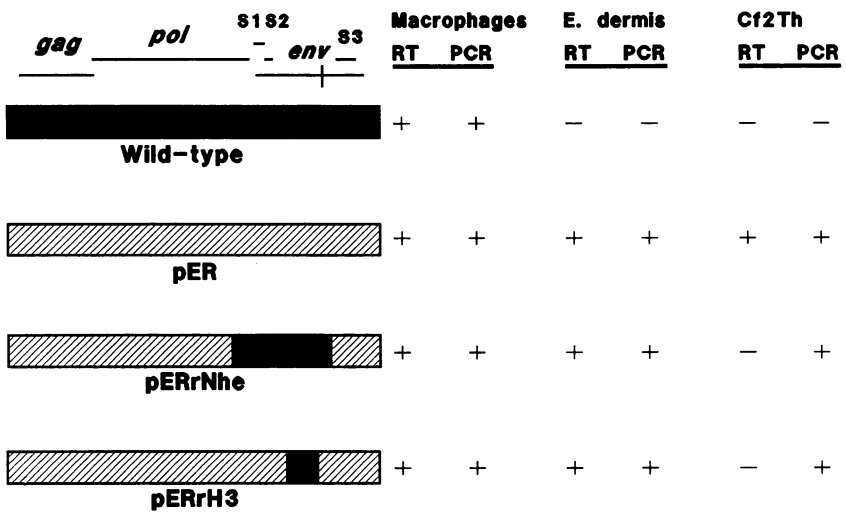


FIG. 6. Schematic diagram of the wild-type replacement proviral clones and a summary of their infectivity phenotypes on equine macrophages, equine dermis, and Cf2Th cells. The solid black regions represent wild-type virus sequences, and the cross-hatched regions represent pER (CL22)-derived regions of the EIAV provirus. Reverse transcriptase (RT) activity is scored as + if it is possible to consistently detect a twofold or greater increase over that of mock-infected cell cultures when cultures are infected with serum-derived wild-type virus particles or particles from pER-, pERrNhe-, or pERrH3-transfected Cf2Th cells. PCR assays were scored as + if it was possible to detect a virus-specific signal by at least 7 days postinfection.

obtained from the passage of cell-free supernatant fluid from transfected Cf2Th cells to equine dermis cells or equine macrophages. A PCR assay was used to detect the presence of provirus in these cells as a measure of viral infectivity. These results indicated that the wild-type molecular clone chimeras pERrNhe and pERrH3 both yield infectious particles from Cf2Th cells that were infectious for equine dermis cells, Cf2Th cells, and equine macrophages. The results that we have obtained for the wild-type chimeras are summarized in Fig. 6. We were able to detect infection of the two wild-type chimeras by PCR assay in all three cell types and were able to detect reverse transcriptase activity in macrophages and equine dermis cells but not Cf2Th cells. It is clear that replacement of all (pERrNhe) or only a portion (pERrH3) of the pER gp90-coding region with sequences that code for wild-type surface protein still allows for efficient replication of these chimeric viruses in equine dermis cells (Fig. 8). The data shown in Fig. 8 compare the relative abilities of EIAV derived from the molecular clones pER, pERrNhe, and pERrH3 and of wild-type virus particles to infect equine dermis cell or equine macrophage cultures. Viruses derived from pER, pERrNhe, and pERrH3 replicate to the same relative levels in equine dermis cells and equine macrophages. Furthermore, these results demonstrate that the time course of virus infection is longer in equine dermis cells (about three times longer is needed to reach peak virus yields) than in equine macrophages and that the yield of virus (determined by reverse transcriptase assay) is also approximately threefold higher in equine dermis cells. Most importantly, only wild-type virus was unable to replicate in equine dermis cells, while virus derived from pER or constructs containing the wild-type surface envelope protein replicated efficiently in equine dermis cells (Fig. 8). Therefore, the tropism of wild-type virus for equine macrophage growth in vitro and restriction in equine dermis cell growth does not map to the surface envelope protein.

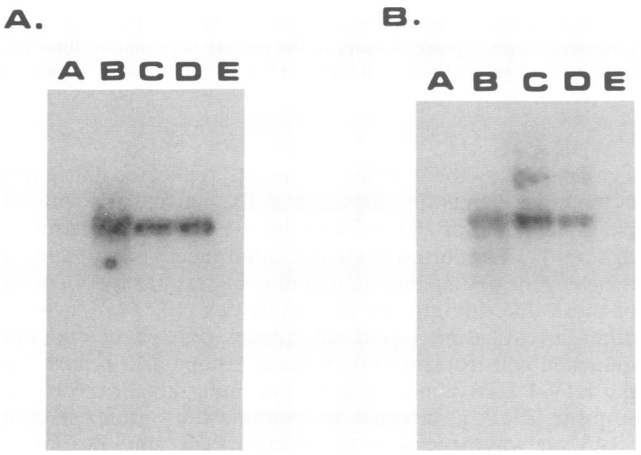


FIG. 7. PCR assay for the detection of infectious virus particles from pER or wild-type replacement clones. Virus particles from Cf2Th cells cotransfected with pSV5neo and either pER or wild-type replacement clones were used to infect equine macrophage cultures (A) or equine dermis cells (B). Lanes A were mock infected with cell-free supernatant fluid from pSV5neo-transformed Cf2Th cells alone, lanes B were infected with cell-free virus particles from pER-transfected cells, lanes C were infected with pERrH3, lanes D were infected with pERrNhe, and lanes E are minus template controls for the PCR assay. Infected cell DNAs were extracted at 7 days postinfection. The virus particle-containing supernatant fluids (lanes B, C, and D) were adjusted so that each inoculum contained 50,000 cpm of reverse transcriptase activity for the macrophage cultures and 200,000 cpm for the equine dermis cell infection.

The observation in Fig. 7 and 8 that EIAV provirus chimeras containing either all (pERrNhe) or a substantial portion (pERrH3) of the wild-type virus surface protein (gp90) gene can productively infect and replicate in equine dermis cells indicates that this cell strain must have a receptor(s) which may be used by the wild-type virus. Therefore, some step subsequent to attachment of wild-type virus to cells is most likely the point at which wild-type virus replication is restricted.

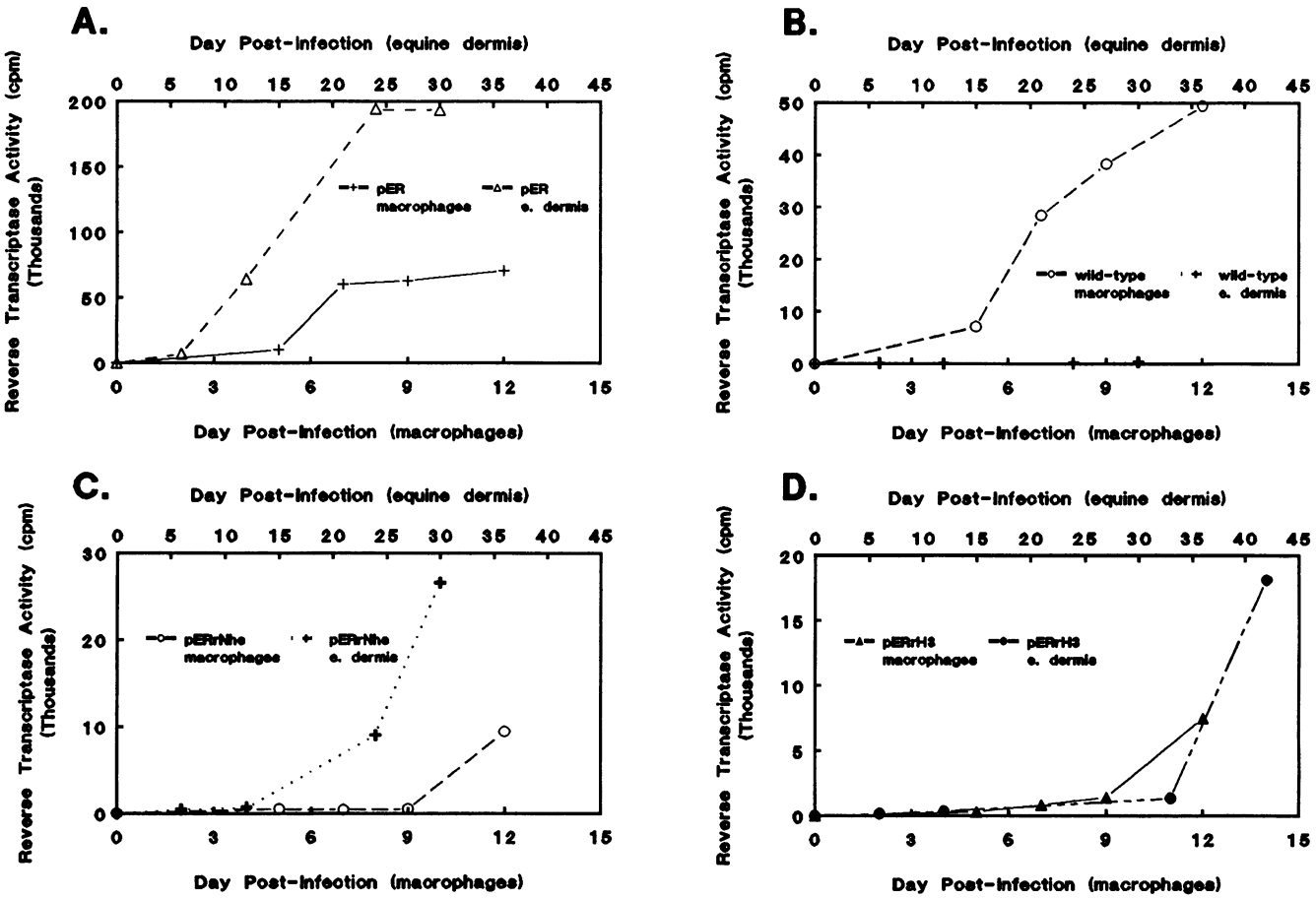


FIG. 8. Comparison of the relative efficiencies of infection of equine dermis cells versus equine macrophages with wild-type EIAV or virus derived from wild-type surface envelope replacement clones. Equine dermis cells or equine macrophages were infected with virus particles derived from pER (A), pERrNhe (C), pERrH3 (D), or wild-type virus (B). Reverse transcriptase activity in the culture supernatant fluid was assayed for up to 45 days postinfection (top x axis) for equine dermis cells or up to 15 days postinfection (bottom x axis) for equine macrophage cultures.

DISCUSSION

In order to examine the restriction of infectivity of the virulent wild-type virus in equine dermis cells, we infected these cells with the virus and then monitored the infection by reverse transcriptase assay and PCR amplification. No reverse transcriptase activity was detected in these cells (Fig. 1), and PCR assay of the infected-cell DNA revealed no signal (Fig. 2), confirming the lack of reverse transcription of the viral genome. The results strongly suggest that the wild-type virus is incapable of performing an early event(s) in the infection of equine dermis cells.

The sequences of EIAV proviral clones reported to date (21, 38, 44) contain in-frame termination codons in either *pol* or *env* open reading frames, accounting for the lack of infectivity of these clones. Since the genetic organization of EIAV is much simpler than those of other lentiviruses, it is also possible that replication-competent EIAV genomes contain additional open reading frames. The genetic organization of CL22 rules out this possibility. Furthermore, since the PCR-derived wild-type sequences represent the genome of a virulent virus, the lack of pathogenicity of CL22 (51) could be attributed to relatively subtle sequence variations between wild-type and cell culture-adapted viruses.

It has long been noted that for HIV, various strains differ significantly in their ability to infect, replicate, and cause cytopathic effects in disparate cells, particularly macrophages and immortalized T-cell lines (14, 53). Several investigators have reported sequence differences, particularly in the *env* gene coding region, that may be responsible for some of these differing growth characteristics (40, 46, 53), while other investigators report differences outside of *env* that influence cell tropism (49). Several groups also report that the HIV-1 LTR is not an important determinant of cellular tropism (23, 35). In order to examine these differences in EIAV, it was necessary to perform PCR amplification of genomic DNA isolated from acutely infected ponies, clone the amplified material, and sequence these wild-type clones. Since the wild-type Wyoming virus has not been biologically cloned, we presume that this strain consists of a heterogeneous population of related viruses, or quasispecies, as described for HIV-1 (15, 17). Sequence differences between individual clones were observed (Fig. 4), but these differences were significantly less than the differences noted between the wild-type sequence and that of CL22.

The LTRs of CL22 and wild-type virus have about a 5% divergence in sequence (most of the differences are in the U3 region) (Fig. 3, Table 1), but it was interesting to note that the wild-type LTR sequence is very similar to the sequence

of a cell culture-adapted clone we obtained previously (pE1-8; data not shown), particularly in the unique feature of having an extra CAAT box motif. Since the virus from which this clone was derived had been passaged through ponies to restore virulence, we were intrigued as to whether this feature may play some role in pathogenicity. However, since the virus was also readapted to equine dermis cell culture, it is clear that such a feature does not segregate with the inability to infect these cells. There are only four differences in the LTR of the wild-type virus that are unique to that virus, indicating that the LTR may not be important in adaptation of the wild-type virus to replication in culture. This property of EIAV may be analogous to the case of HIV-1, in which the LTRs of a macrophagetropic and T-lymphocyte-tropic virus were unable to influence cell tropism when ligated to the viral backbone of a T-lymphocyte-tropic strain (23).

The large number of coding differences in gp90 and gp45 was not surprising; sequence divergence in the envelope protein and its role in antigenic variation have been previously described for EIAV (32) as well as several other lentiviruses, including HIV-1. The extent of differences found between the cell culture-adapted CL22 and the wild-type sequence was, however, much greater than that reported among EIAV isolates derived from the original Malmquist strain (27) and passaged in ponies (0.6 to 1.4% nucleotide divergence; 1.3 to 3.4% amino acid divergence) (31, 33). For gp90 there is a 5.57% nucleotide sequence divergence between the wild type and CL22, resulting in an 11% amino acid sequence divergence. The wild-type virus surface protein is 5 or 6 amino acids shorter than the CL22 surface protein (438 to 439 versus 444 amino acids). Three domains have been identified by monoclonal antibodies as neutralizing epitopes C, D, and E (3, 19), as indicated in Fig. 5. All three of these epitopes contain significant coding differences. The region defined as the principal neutralizing determinant (3) was found to contain a deletion of 49 nt which was replaced with an insertion of 31 nt, preserving the reading frame but substituting 11 amino acids for the 17 encoded by CL22. It was interesting to note that in one region designated hypervariable by Payne et al. (32), 10 of 14 amino acids were different; among the four individual clones which contained this region, all four contained the identical sequence.

Several of our wild-type Wyoming virus envelope clones (Fig. 3) contain one or more in-frame termination codons, indicating that these were derived from defective proviruses in the infected horse. We feel that these defective templates actually occur in the infected horse and are not likely to be a PCR-generated artifact, because we have observed defective templates only in the *env* gene region (predominantly in gp45). Miller et al. (28) have suggested that lentivirus transmembrane proteins contain an amphipathic α-helical peptide which may be cytolytic to both mammalian and bacterial cells. If such a peptide were expressed during the cloning process in bacterial hosts, this could result in a selection for clones which contain mutations preventing expression of these cytolytic sequences. While it certainly may be that cloning does select for these defective sequences, we would argue that in order for such sequences to be cloned, they must be present in the mononuclear cell DNA of infected horses. Recent reports by Kusumi et al. (26) using similar techniques of PCR amplification, followed by cloning and sequencing of HIV-1 envelope genes, have indicated that at least 50% of the virus population in circulating blood cells of infected patients are defective. It has been suggested that defective HIV proviruses may play a role in the pathogenesis of AIDS (50). Additionally, other studies have shown that there is a greater percentage of defects in envelope gene sequences having the G→A hypermutation (48), such as that described for these clones. Further, since at least one of our large *env* clones, pFD, does not contain any obvious defects that would prevent gp45 expression (including the putative cytolytic peptide), we are confident that our clones accurately reflect sequences found in circulating mononuclear cells of EIAV-infected animals.

From the sequence data summarized above, it seemed most likely that differences in the outer membrane envelope gene could be responsible for the restriction of wild-type virus in equine dermis cells. Therefore, we constructed a series of chimeric clones containing wild-type sequences on the pER (derived from CL22) background. We first replaced the 750-bp *Hin*dIII fragment in pER with its wild-type counterpart (pERrH3). This region contains both of the highly divergent regions of gp90 discussed above. We also replaced the 2.88-kb *Nhe*I fragment from pER with its wild-type counterpart (pERrNhe). This region contains all of the outer membrane envelope sequences, the second exon for S1 (the first exon has been shown not to be critical for *tat* activity [29, 45]), and the coding region for S2. Therefore, if differences in any of these genes are responsible for the restriction of growth of the wild-type virus in equine dermis cells, virus from this chimeric clone (pERrNhe) should be unable to infect these cells. However, when virus harvested from Cf2Th cells transfected with either pERrH3 or pERrNhe was used to infect equine dermis cells, it was clear that virus from both chimeric clones was able to replicate in this cell type (Fig. 7 and 8). A comparison of the PCR assay signals and the reverse transcriptase activities of these chimeras compared with those of pER-derived virus suggests that the wild-type surface envelope protein allows for efficient virus replication in equine dermis cells and is not responsible for the dramatic restriction of wild-type virus replication in these cells. Several reports have described the restriction of replication of some isolates of HIV-1 replication in certain cell types, and these properties map to the envelope gene region (46, 53). Viral entry and/or uncoating may also be a major determinant of viral tropism (4, 23). Viral entry is thought to be the result of a complex interaction between host and viral components, in addition to receptor-envelope binding. Other cell proteins may be involved and may be necessary to interact with the receptor proteins or may facilitate the internalization and uncoating of virus in the host cell. For EIAV, specific receptors have not yet been identified. Even with the identification of specific receptors, it is clear that much concerning the infection process of EIAV remains to be determined. We are continuing our analysis of EIAV chimeras to determine which portion(s) of the wild-type Wyoming virus genome is responsible for the restriction of growth in vitro.

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