Genetic Characterization of Two Phenotypically Distinct North American Ovine Lentiviruses

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Ovine and caprine lentiviruses are closely related genetically and antigenically although the diseases that these viruses cause in their respective host animals can vary greatly. In sheep, syndromes consist primarily of interstitial pneumonia with rare occurrences of arthritis and encephalitis, whereas in goats, the disease expresses mainly as arthritis in adult animals with rare cases of encephalitis in newborns. Experimentally, viruses from either sheep or goats can infect animals of the reciprocal species and many field strains of ovine lentivirus have biological properties similar to those of caprine viruses. However, a molecular correlation for the phenotypic differences between ovine and caprine lentivirus strains is unknown. To investigate this, we examined genetic characteristics of two phenotypically distinct North American ovine lentiviruses. Nucleotide sequence analysis of the envelope regions from virus strains 85/34 and 84/28 showed that despite significant biological differences, these viruses are closely related to each other and are genotypically more homologous to caprine arthritis-encephalitis virus (CAEV) than to visna virus of sheep. Furthermore, analysis of the nucleotide substitutions in their env regions indicated that when differences between the two ovine viruses and CAEV were found, the changes often resulted in nucleotides homologous with visna virus. These results suggest that the two field strains of ovine lentivirus may have originated from a cross-species infection of sheep by a CAEV-like virus and, evolution of their genomes toward that of ovine lentivirus may be reflective of adaptation of these viruses to the new ovine host.
These findings raise questions about the origins of phenotypically distinct virus strains and whether cross-species infections may, in part, account for the diversity of viruses obtained from naturally infected animals. Experimentally, CAEV and visna virus have each been shown to infect both sheep and goats. Banks et al. (1983) found that lambs inoculated with CAEV developed both arthritis and antibodies to CAEV. Similarly, goats inoculated with OPPV replicated the virus and developed arthritis and antiviral antibodies (Banks et al., 1983). However, it is not known whether cross-species infection of ovine and caprine lentiviruses is a common occurrence.

Lairmore et al. (1987) reported the isolation of two infectious and pathogenic OvLVs from naturally infected North American sheep. Early studies showed that strain 85/34 was lytic in cell cultures, replicated to high titers in goat synovial membrane (GSM) cells, and induced severe lymphoproliferative disease in expanded by cultivation in Minimum Essential Medium to high titers in goat synovial membrane (GSM) cells, (Klevjer-Anderson and Cheevers, 1981). This cell line was that strain 85/34 was lytic in cell cultures, replicated membrane from a colostrum-deprived newborn goat infected North American sheep. Early studies showed tained originally from cultured explants of carpal synovial

In vivo pathologic (Lairmore et al., 1987; Concha-Bermejillo et al., 1995; Woodward et al., 1996; Lairmore et al., 1988). These findings led to the characterization of strain 85/34 as type I virus and strain 84/28 as type II. While phenotypic differences between these two OvLV strains have been well characterized, to date genetic information from these isolates has not been reported. In this study we obtained partial nucleotide sequences from the env genes of OvLVs 85/34 and 84/28 and compared these sequences to the corresponding env regions of known ovine and caprine lentiviruses to determine their genetic relationship to visna virus and CAEV. This region of the viral genome was chosen because it has the highest sequence diversity among lentivirus strains and is thought to contain determinants of both host cell tropism and pathogenicity (Braun et al., 1987; Crane et al., 1988). These data showed the two OvLVs to share 97.5% nucleotide sequence homology to each other, 72.7% homology with CAEV, and 67% homology with visna virus, somewhat equidistant between the two prototypes with slightly more homology to CAEV than visna. However, analysis of nucleotide substitutions in the env sequences of these viruses indicated that when differences between the two OvLVs and CAEV were observed, the changes were often homologous with those of visna virus. These results suggested that the two field strains of ovine lentivirus may have originated from a cross-species infection in sheep with a CAEV-like virus and, during persistent infection in sheep, their genomes evolved gradually toward that of ovine lentivirus. This type of genetic selection may be reflective of adaptation of these viruses to the new ovine host and perhaps to the unique type of disease to which this species succumbs.

Methods

Viruses and cell culture

OvLV strains 85/34 and 84/28 were isolated from two American sheep that had lymphoid interstitial pneumonia (LIP) and both LIP and ovine pulmonary carcinoma, respectively (Lairmore et al., 1987, 1988). These viruses were passaged several times in goat synovial cell cultures and plaque purified (Lairmore et al., 1987). Stocks of the two OvLV strains were subsequently propagated in goat synovial membrane cell cultures. Infectivity titers of these stocks were determined to be approximately 10^6 and 10^5 TCID50/ml for OvLV strains 85/34 and 84/28, respectively, when titrated in GSM cell cultures.

Goat synovial membrane (GSM) cells had been obtained originally from cultured explants of carpal synovial membrane from a colostrum-deprived newborn goat (Klevjer-Anderson and Cheevers, 1981). This cell line was expanded by cultivation in Minimum Essential Medium (MEM) + 10% fetal bovine serum (FBS) and stored in liquid nitrogen. Typical monolayer cultures were passaged at 1:3 split ratios and used for 7–10 passages. For inoculation with virus, media was aspirated from GSM monolayers and virus added at a m.o.i. of 0.10 in MEM + 2% FBS. After 3 hr at 37°C, inoculum was removed and replaced with fresh MEM + 5% FBS.

Amplification of OvLV DNA

For amplification of viral env DNA from OvLVs 85/34 and 84/28, GSM cells infected for 1 week with either virus were lysed in buffer containing 10 mM Tris–HCl, pH 8.3, 50 mM MgCl₂, 1 mg/ml gelatin, 20 mM dithiothreitol (DTT), and 7 μM SDS. Cell lysates were then digested with 50 μg/ml proteinase K and incubated for 1 hr at 37°C. The proteinase K was inactivated by heating to 86°C for 10 min. These lysates were stored at −80°C for subsequent PCR reactions. PCR amplification of OvLV DNA has been previously described (Chebloune et al., 1996a). Briefly, two sets of oligonucleotides specific to the env gene of CAEV strain CO, according to the published sequence of Salterelli et al. (1990), were used. Oligonucleotide primers 5’ CAGATAACATGCCTAACTGGAAAG 3’ and 5’ CTGTTGTCATTTCTCAAGCCATCG 3’ are complementary to bases 6026–6052 and 8605–8632, respectively, and primers 5’ GGCCCTCTGGCCAAATGGAG 3’ and 5’ AGCTTCCACTCGAGCCACTCG 3’ are complementary to bases 6475–6494 and 8115–8135, respectively. This nested set of oligonucleotide primers amplifies a 1.6-kb fragment of the CAEV env gene. PCR reactions were performed in the presence of 0.25 mM of each deoxynucleotide triphosphate, 500 nM of each oligonucleotide primer, 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 8 mM MgCl₂, 20 μl of cell lysate containing DNA (equivalent to approximately 10^5 cells) and 2.5 units of Taq DNA polymerase (Perkin/Elmer). The reactions were per-
formed in 100 μl final volume covered with 50 μl of mineral oil. Samples were denatured at 94° for 3 min, then subjected to 35 cycles of amplification (92° for 1 min, 48° for 1.5 min, and 68° for 2.5 min in a Perkin/Elmer thermocycler). Reactions were extended for 15 min at 68° before storage at 4°.

Subcloning and sequencing of OvLV envelope DNA

PCR-amplified env DNA from OvLVs 85/34 and 84/28 was purified on agarose gels and subcloned using the pGEM-T Vector system (Promega, Madison, WI) as recommended by the manufacturer. Positive plasmid constructs were screened by restriction enzyme digestion and Southern hybridization with a [α-32P]dCTP labeled 3-kb restriction fragment of the CAEV env gene isolated from pCAEV (Pyper et al., 1986). After selection of positive clones, amplified plasmid DNA was purified from Escherichia coli using the Qiagen plasmid purification system (Qiagen Inc., Chatsworth, CA). Purified plasmid DNA containing the OvLV env fragments were sequenced by the primer-directed dideoxy chain termination method using Sequenase version 2.0 DNA sequencing kit. (United States Biochemicals, Cleveland, OH).

Genetic and phylogenetic analyses

The 1.6-kb env DNA sequences obtained from OvLVs 85/34 and 84/28 were analyzed along with the corresponding env sequences derived from the Icelandic MVV strain 1772 (Braun et al., 1987), the British MVV strain EV1 (Sargan et al., 1991), and the South African MVV strain SA-OMVV (Querat et al., 1990), as well as the North American caprine isolates CAEV strain CO (Saltarelli et al., 1990) and CAEV strain 63 (Knowles et al., 1991). North American OvLV env sequences, SH11 and SH8, were obtained from a previous study (Chebloune et al., 1996a). The env region of bovine immunodeficiency virus (BlV) (Garvey et al., 1990) was included in our analyses as an out group.

Sequence alignments were performed using the Genetics Computer Group (Devereux et al., 1984) software package available on the silicon graphics work station at KUMC. Nucleotide sequences from the above strains were aligned by PILEUP using the progressive alignment method (Feng and Doolittle, 1987). Evolutionary distances (number of nucleotide substitutions) were estimated using one-parameter (Jukes and Cantor, 1969) and two-parameter (Kimura, 1980) methods. Evolutionary distances of the derived amino acid sequences were estimated using the Kimura protein distance method (Kimura, 1983). Phylogenetic trees, assuming BlV as the out group, were constructed using programs available through PHYLIP (Phylogeny Inference Package) version 3.5c, distributed by Joe Felsenstein at the University of Washington, Seattle Department of Genetics (Felsenstein, 1989). Bootstrapped data sets (1000 resamplings of the original aligned sequences) were generated using SEQBOOT. Phylogeny estimates for each data set were then obtained by the DNAPARS program which carries out unrooted parsimony to find the maximum parsimonious tree for each resampling. The majority rule consensus tree from this analysis was then found using the CONSENSE program. The percentage of nucleotide substitutions in the OvLV env regions which deviated from the sequence of CAEV strain CO but were homologous to those of visna sequences were scored manually from the aligned sequences shown in Fig. 2.

RESULTS

The env regions of OvLV strains 85/34 and 84/28 are closely homologous to each other

To amplify viral DNA from GSM cells infected with either of the two OvLVs, we tested oligonucleotide primers specific to the env regions of both MVV and CAEV. Surprisingly, whereas attempts to amplify DNA using primers specific to the MVV strain 1772 genome were unsuccessful, PCR amplification using primers specific to the CAEV strain CO env region produced DNA products of the predicted size (data not shown). PCR reactions using the CAEV env-specific primers produced 1.6-kb PCR products which were subsequently cloned into the pGEM-T vector and their respective nucleotide sequences determined using standard sequencing methods. When aligned with each other, the env regions of the two viruses were found to have 97.5% sequence homology (Table 1). These results indicated a close relationship of these viruses to each other and suggested that they originated from a common ancestral strain.

The env regions of OvLVs 85/34 and 84/28 are more homologous with CAEV than visna virus

The genomes of three strains of ovine visna virus were reported to have approximately 80% homology with each other, while the two prototypic strains of caprine viruses CAEV-CO and CAEV-63 share about 85% sequence homology (Saltarelli et al., 1990; Braun et al., 1987; Knowles et al., 1991). However, when compared to each other, the known ovine and caprine lentiviruses have only about 60% sequence identity in their env regions (Knowles et al., 1991) (Table 1). To understand their genetic relationship with previously sequenced ovine and caprine lentiviruses, we compared the env regions of OvLVs 85/34 and 84/28 with the corresponding sequences of two caprine isolates CAEV-CO and CAEV-63, two other North American OvLVs (SH11 and SH8), and three ovine visna virus strains, Icelandic MVV-1772, British MVV-EV1, and the South African SA-OMVV. The mean homology between the env sequences of OvLV strains 85/34 and 84/28 and those of CAEV was 72.7% with a range of 72.0–73.4%. However, compared to the three MVV strains, the OvLV
sequences shared 67% homology with a range of 65.6–68.0% (Table 1). Similar analysis of the amino acid sequences also showed a slightly higher homology of the OvLVs with CAEV (70.7%) compared to visna viruses (68.6%). The percentages of sequence homologies in the env regions of these ovine and caprine lentivirus strains were used to derive a phylogenetic tree (Fig. 1). This analysis grouped OvLVs 85/34 and 84/28 in a cluster with CAEV strains CO and 63 and OvLVs SH11 and SH8, and distant from the cluster of MVV-1772, SAOMV, and EV1. These results suggested a closer evolutionary relationship of the OvLVs with CAEV than visna virus.

Nucleotide substitutions in the env genes of OvLVs 85/34 and 84/28 are homologous with those of visna virus

When we examined the aligned env sequences shown in Fig. 2, we observed that many nucleotide substitutions in the OvLV sequences which distinguished these viruses from CAEV-CO were homologous with those from visna viruses MVV-1772, SAOMV, and/or EV1. Quantitation of the number of these types of substitutions in the env sequence of OvLV 85/34 showed that approximately 43% of the total base changes in this sequence different from the sequence of CAEV-CO were homologous to MVV-1772 (Table 2). Similarly, 43–44% of the total base changes from CAEV-CO in the env sequence of OvLVs 84/28 and SH11 were homologous to MVV-1772. Compared to each visna virus sequence individually, about 42–44% of the nucleotide changes in the OvLV sequences were homologous to a specific visna sequence (Table 2). With one exception (SH11 versus EV1) all of the percentages were above the 33% that would be expected if these changes were occurring randomly. These results are suggestive of a state of evolution in these OvLV genomes toward the genome of visna virus in sheep.

**DISCUSSION**

We have demonstrated that two phenotypically distinct field strains of North American ovine lentivirus are closely related to each other genetically and that their env regions are more homologous to that of the caprine lentiviruses than to the classical visna lentiviruses of sheep. However, analysis of nucleotide substitutions in the env genes of the two viruses indicated that when differences between the two and CAEV were examined, the changes were often homologous with those from visna virus genomes. These findings suggest that the two North American OvLVs may have originated from CAEV or a CAEV-like virus, possibly by natural cross-species transfer of lentivirus from goat to sheep, and that subsequent virus adaptation in sheep led to their evolution toward the genome of visna.

Following their isolation from naturally infected sheep, OvLV strains 85/34 and 84/28 were passaged several times through goat synovial cells, and the env sequences reported in this study were obtained from GSM cells infected in vitro using oligonucleotide primers specific for CAEV. Therefore, we cannot exclude the possibility that previous cultivation of these viruses in goat cells and our cloning techniques resulted in selection of variants more closely related to goat viruses. However, multi-
 Phylogenetic analysis of ovine/caprine lentiviruses has not changed their phenotypes as both viruses have remained pathogenic in sheep and have retained their distinct biological properties (Lairmore et al., 1987; Concha-Bermejillo et al., 1995; Lairmore et al., 1988). Our attempts to amplify OvLV env DNA using primers homologous to MVV sequences failed. Thus, as with our previous study of OvLV DNA from naturally infected farm sheep (Chebloune et al., 1996a), only primers specific to CAEV were successful in amplifying the OvLV DNA. In addition, oligonucleotide primers specific to the env sequence of OvLV 85/34 reported here readily amplified viral DNA from peripheral blood mononuclear cells of sheep infected with this virus (Chebloune et al., unpublished data). If the env sequences amplified in this study are from rare variants more closely related to goat lentiviruses, these variants are not strongly selected against by in vivo passage of the OvLVs in sheep. Therefore, we believe that the sequences reported here are an accurate representation of naturally occurring North American ovine lentiviruses and are relevant to this study.

The biological characteristics of OvLV strains 85/34 and 84/28 have been extensively characterized both in vitro and in vivo (Lairmore et al., 1987; DeMartini et al., 1993; Concha-Bermejillo et al., 1995; Lairmore et al., 1988). Strain 85/34 was found to be highly cytopathic in cell culture and pathogenic in infected animals, while strain 84/28 replicated poorly and nonlytically in vitro and produced little in vivo pathology. Based on these past studies, strain 85/34 was reported to be a type I (visna-like) virus and strain 84/28 was characterized as type II (CAEV-like) (Lairmore et al., 1987, 1988), alluding to the classification of HIV strains as “fast/high” and “slow/low” viruses. We found that despite their distinct biological characteristics, the two OvLVs are closely related genetically, with approximately 97.5% sequence homology within their env regions. Although the genetic determinants for phenotypic differences between OvLVs 85/34 and 84/28 remain unknown, it is possible that either these determinants lie outside of the env region reported here or that very few nucleotide changes can result in the dramatic biological differences of these viruses.

A recent study by Leroux et al. found that viruses obtained from several sheep in France were more homologous with CAEV than visna virus in phylogenetic analyses of nucleotide sequences in their pol genes (Leroux et al., 1995). Similarly, our results showed that when compared to the published sequences of three ovine visna virus strains MVV-1772, -SA-OMVV, and -EV1, and two caprine strains CAEV-CO and -63, the env sequences of OvLVs 85/34 and 84/28 had a higher percentage of homology to CAEV than visna virus with mean intersolate homologies of 72.7 and 67.0%, respectively. These results also agree with our recent study of naturally infected North American sheep (Chebloune et al., 1996a). Thus, natural infection of sheep with lentiviruses more closely related to CAEV than visna virus may be a common occurrence in different areas of the world.

FIG. 1. Phylogenetic tree based on the 1.6-kb env region of ovine and caprine lentiviruses. Unrooted maximum parsimony trees based on 1000 bootstrap resamplings of the aligned sequences (using SEQBOOT) were constructed by DNAPARS. The majority rule consensus tree from this data set was then selected by CONSENSE. Branch lengths are proportional to the estimated number of nucleotide substitutions, and bootstrap probabilities (in percentage) are given beside each internal branch. The scale bar is equal to approximately 5% nucleotide divergence.
FIG. 2. Nucleotide sequences of the env regions of OvLV strains 85/34 and 84/28 and known ovine and caprine lentiviruses. Nucleotide sequences within the 1.6-kb env region were aligned using the PILEUP multi-alignment program of GCG. From top to bottom are the corresponding sequences of the North American caprine lentivirus isolates, CAEV-CO and CAEV-63, the North American OvLVs SH11, 84/28, and 85/34, and the visna virus strains MVV-1772, -SA-OMVV, and -EV1. (0) homologous nucleotides; (−) deleted nucleotides. Regions of sequence conservation (C1), variability (V1, V2, and V3), and hyper variability (HV1 and HV2), as previously reported (Pyper et al., 1986), are indicated above the CAEV-CO sequence. Asterisks placed below the aligned sequences indicate nucleotide substitutions in the OvLV 85/34 sequence that are different from CAEV-CO but homologous to MVV-1772.
Interestingly, with the sheep reported in our previous study, infectious virus could not be isolated from cultured macrophages containing viral DNA (Chebloune et al., 1996a). The env sequences from the DNAs of SH11 and SH8 were found to be about 84% homologous with CAEV.

In contrast, the env regions of OvLV strains 85/34 and 84/
28 are about 72.7% homologous with CAEV. One possible explanation for differences in homology between these two pairs of ovine viral DNAs to CAEV may be that, while viral DNA from cells of sheep SH11 and SH8 appeared to be inactive, strains 85/34 and 84/28 are infectious and pathogenic in sheep. Thus, the higher sequence divergence of OvLVs 85/34 and 84/28 from CAEV may be reflective of their state of adaptation in sheep. A surprising observation from analysis of the nucleotide substitutions in the env sequences of OvLV strains 85/34, 84/28, and SH11 was that when differences between these viruses and CAEV were found, the changes were often homologous with nucleotides from corresponding visna virus sequences. This observation suggests that

![Diagram of nucleotide sequences for CAEV, OvLV, and SH11 strains](image-url)
the OvLVs, which are genetically more similar to CAEV than visna virus, were in a state of evolution toward the genome of visna. Whether evolution toward the visna virus genome in the env genes of OvLV in sheep correlates with host-specific differences between sheep and goats is unknown.

While our results present only indirect evidence, it is tempting to speculate that these North American strains of OvLV originated from a cross-species transmission of CAEV or CAEV-like virus from goat to sheep and this type of genetic selection of the OvLV genome may be reflective of the adaptation of these viruses to sheep. We cannot exclude the possibility that the OvLVs evolved independently in North American sheep; however, it is surprising that these viruses would have env sequences more similar to CAEV than to the classic strains of sheep visna virus. Another possibility is that strains of North American CAEV originated from lentiviruses of sheep. This could explain the close relationship of the OvLVs to CAEV, but again it is surprising that these ovine lentivirus strains would be so divergent from MVV. The true origins of these OvLV strains remain unknown, but further investigation of the genetic and phenotypic characteristics of ovine/caprine lentivirus field strains may provide insights into the natural occurrence of cross-species lentivirus transmission and how this may affect the emergence of new virus strains.

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