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Sequence Homology between Cloned Caprine Arthritis Encephalitis Virus and Visna Virus, Two Neurotropic Lentiviruses

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Caprine arthritis encephalitis virus (CAEV) is an exogenous, nononcogenic retrovirus which causes neurological disease and crippling arthritis in goats. A complete CAEV genome was cloned from unintegrated viral DNA in two fragments of 9.4 and 0.4 kilobases in length, respectively. The biological activity of these clones was tested by ligation of the fragments followed by transfection onto goat synovial membrane cells; infectious virus was recovered. Cloned CAEV and visna virus, a related neurotropic virus of sheep, were compared by heteroduplex and molecular hybridization analyses. These data demonstrated that the greatest overall conservation of nucleotide sequences occurred in the *gag* and *pol* gene regions and two smaller regions, *sor* and the putative *tat* gene. The region of greatest divergence occurred in the *env* gene and, in particular, was localized primarily in the region coding for the glycosylated outer membrane protein. These findings and the recently demonstrated genetic relationship of visna virus, CAEV, and human T-cell lymphotropic virus type III, the etiologic agent of the acquired immune deficiency syndrome, may have important implications concerning the biological properties of these related viruses for human and veterinary medicine.

The lentiviruses are exogenous, nononcogenic retroviruses which cause persistent infections coincident with chronic progressive diseases in their hosts. Two morphologically related lentiviruses, visna virus in sheep and caprine arthritis encephalitis virus (CAEV) in goats, produce slowly progressive inflammatory lesions in the brain, joints, and lungs of infected animals (4–7, 10, 11, 19, 32). Sheep develop mainly the pneumonic form of the disease complex, and this leads to neurological complications in some animals (19, 32). In goats, the expression of disease is age dependent, with neurological disease occurring mainly in young animals (4–7). These viruses are widespread in sheep and goat populations in many parts of the world and, therefore, are of economic importance.

The target cells for both CAEV and visna virus in the infected animal are the peripheral blood monocytes, although little virus is produced until the monocyte matures into a macrophage (1, 29, 31). Both viruses cause cell fusion (multinucleated giant cells) and cytolytic events in infected cells in culture. They are also serologically related and share nucleic acid homology over their entire genome, with the strongest homology in the *gag-pol* region (15, 16, 34, 36).

A nononcogenic human retrovirus, human T-cell lymphotropic virus type III (HTLV-III), the etiologic agent of the acquired immune deficiency syndrome, is also cytolytic in vitro, causing extensive cell fusion and cell death. In addition, HTLV-III infects cells of the immune system, in particular a subset of helper T-lymphocytes (14, 33, 44). HTLV-III appears to be neuropathic and is believed to be the cause of the acquired immune deficiency syndrome encephalopathy, which includes dementia and motor impairment (38). In addition, HTLV-III has been shown to morphologically resemble and to share nucleotide sequence homology with visna virus (17).

The genetic relationship between HTLV-III and visna virus (17) has raised considerable interest in these neurotropic lentiviruses and in the extent and localization of nucleotide sequence homology within this group. We therefore derived and report the structure of a biologically active clone of CAEV. A molecular clone of another strain of CAEV had previously been reported, but this clone did not have biological activity (45); the clones reported here are the first infectious lentivirus clones to be isolated. We have used these clones to further define regions of nucleic acid homology between CAEV and a molecular clone of visna virus (25) by heteroduplex mapping and molecular hybridization analyses.

Hirt supernatant DNA isolated from CAEV (strain CO)-infected goat synovial membrane cells was digested with *Hin*dIII. This restriction enzyme cleaves CAEV Hirt supernatant DNA at 0.2, 9.6, and 10.0 kilobases (kb) from the 5′ end of the linear DNA (34) (Fig. 1A). The sites at 0.2 and 10.0 kb are within the long terminal repeats (LTRs). The *Hin*dIII-digested CAEV DNA was ligated to doubly-digested (*Hin*dIII and *Bam*HI) Charon 35 DNA (23). The Charon 35 DH-1 vector-host system, effectively a *recA*$^-$ *recBC*$^-$ system, was used to minimize recombination and deletion events occurring in the bacterial cell (23).

From multiple positive clones, two distinct types of clones were obtained which hybridized to CAEV cDNA. Clone 9.4 contained the 9.4-kb *Hin*dIII fragment, and clone 0.4 contained the 0.4-kb *Hin*dIII fragment (Fig. 1A). Similar restriction endonuclease digestion patterns are generated from Hirt supernatant DNA from CAEV-infected goat synovial membrane cells and the 9.4-kb *Hin*dIII insert from the Charon 35 clone (Fig. 1B). Small probes made from the ends of the cloned DNA hybridized to the expected restriction fragments of Hirt supernatant DNA (data not shown).

To ascertain the origin of the 0.4-kb *Hin*dIII fragment, ^{32}P-labeled probe was prepared from the 0.4-kb fragment (12, 13). Hirt supernatant DNA was digested with restriction endonucleases, and identical sets were electrophoresed in a 1.2% agarose gel and transferred to nitrocellulose. One set of transfers was hybridized with representative probe made

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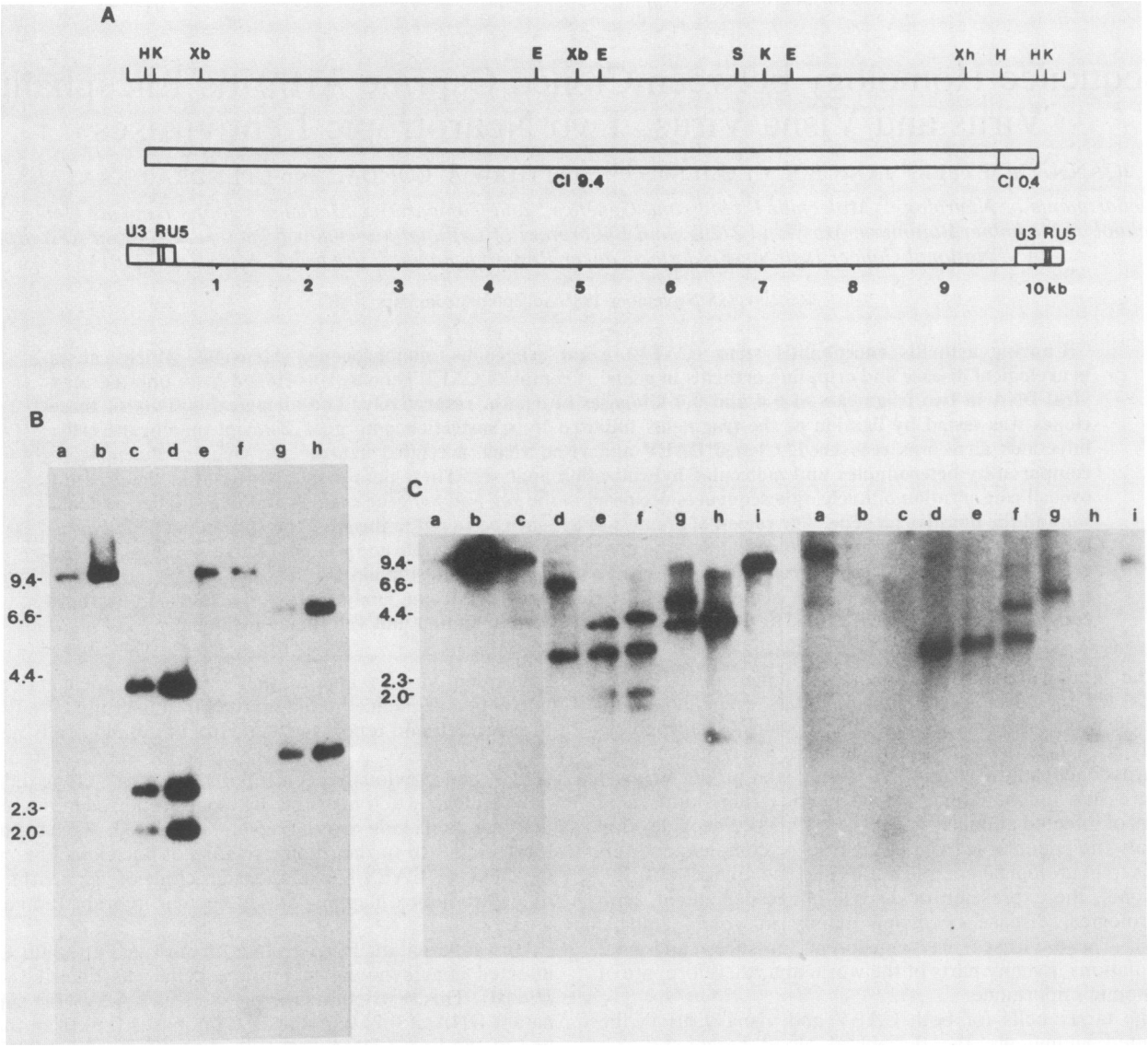

FIG. 1. Characterization of lambda clones 9.4 and 0.4 (A) Physical map of restriction endonuclease sites in CAEV Hirt supernatant DNA and the locations of clones 9.4 (Cl 9.4) and 0.4 (Cl 0.4). Abbreviations and sites of cleavage for the restriction endonucleases are as follows: E (*Eco*RI) at 4.5, 5.2, and 7.3 kb; H (*Hin*dIII) at 0.2, 9.6, and 10.0 kb; K (*Kpn*I) at 0.3, 7.0, and 10.1 kb; S (*Sma*I) at 6.7 kb; Xb (*Xba*I) at 0.8 and 4.95 kb; and X (*Xho*I) at 9.2 kb. (B) Comparison of restriction endonuclease digestions of CAEV Hirt supernatant DNA and clone 9.4 DNA. DNA was digested with restriction endonucleases, subjected to electrophoresis in a 1.2% agarose gel, and transferred to nitrocellulose (41). The DNA transfer was hybridized with ^{32}P-labeled representative cDNA prepared from CAEV virion RNA. Lanes a, c, e, and g contain Hirt supernatant DNA. Lanes b, d, f, and h contain clone 9.4 DNA. Lanes a and b, *Hin*dIII digest; lanes c and d, *Hin*dIII-*Eco*RI digest; lanes e and f, *Hin*dIII-*Xho*I digest; and lanes g and h, *Hin*dIII-*Sma*I digest. (C) Hybridization of restriction endonuclease-digested DNA with ^{32}P-labeled representative probe and with probe prepared from clone 0.4. Except when noted, lanes contain Hirt supernatant DNA cut with the following enzymes: a, undigested; b, clone 9.4 DNA digested with *Hin*dIII; c, *Hin*dIII; d, *Kpn*I; e, *Eco*RI and *Kpn*I; f, *Eco*RI; g, *Xba*I; h, *Xba*I and *Xho*I; i, *Xho*I; left, hybridization with representative cDNA probe; right, hybridization with probe made from clone 0.4.

from CAEV virion RNA, and one set was hybridized with probe made from clone 0.4 (Fig. 1C). If clone 0.4 contained LTR sequences as well as some unique 3′ sequences, it would be expected to hybridize to restriction fragments generated from both ends of the CAEV Hirt supernatant DNA. As expected, the small probe did not hybridize to clone 9.4 DNA, but did hybridize to the appropriate restriction fragments from Hirt supernatant DNA (Fig. 1C). These data demonstrate that clone 0.4 contains the small U3-containing *Hin*dIII fragment. This has been confirmed by DNA sequence analysis (data not shown). We believe that the 0.4-kb fragment was ligated to other fragments (either stuffer fragments or fragments generated from the digested Hirt supernatant DNA) in such a way that a piece at least 9.0 kb long was inserted into the vector and therefore could be packaged as a viable phage. The LTRs at the ends of CAEV linear double-stranded DNA are about 500 base pairs long, and the *Hin*dIII sites at 0.2 and 10.0 kb on the restriction map (Fig. 1A) lie within these LTRs. Thus, the two clones together appear to contain all the genetic information of the virus.

To verify that an infectious clone could be regenerated,

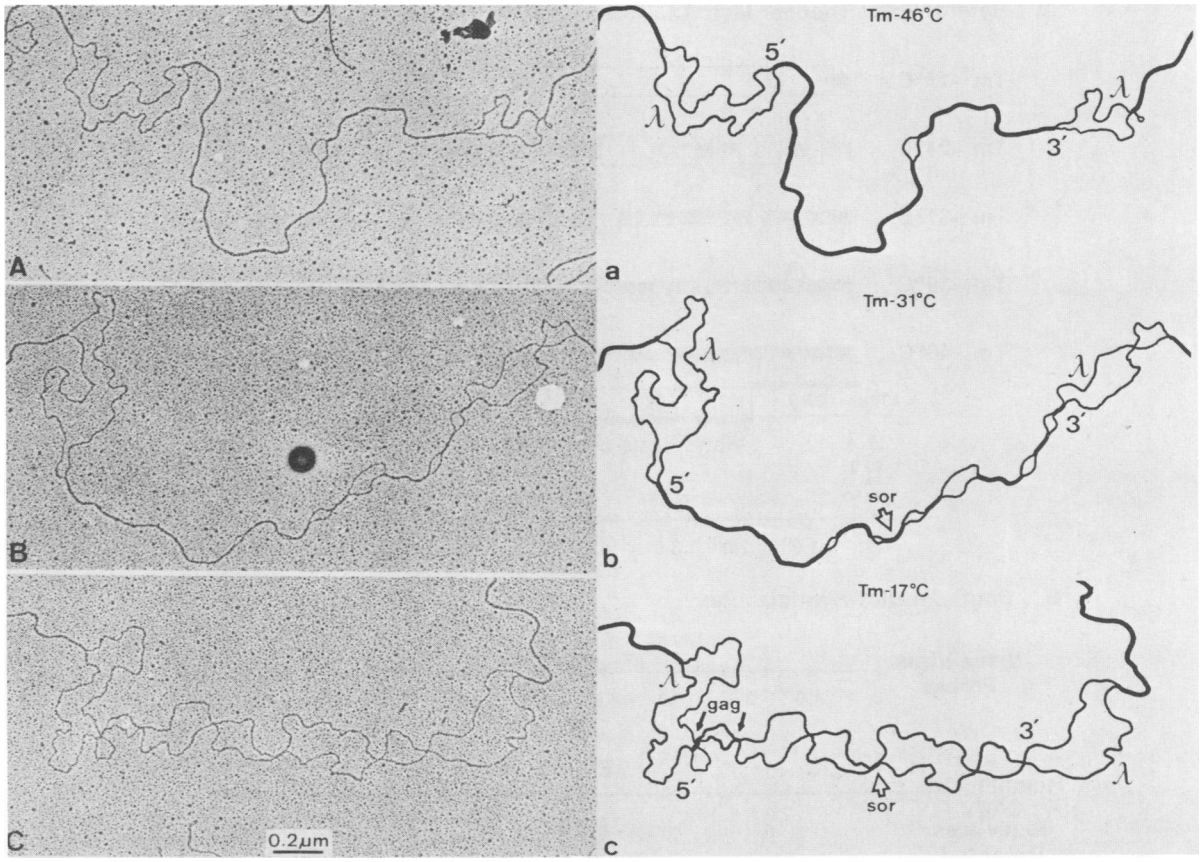

FIG. 2. Heteroduplex analysis of CAEV (clone 9.4) and visna virus strain 1514 (25) clones under varied stringencies. Heteroduplexes were prepared by the method of Davis et al. (9), with modifications as described in reference 17. For thermal melt analysis the concentration of formamide was varied from 20 to 60% (8, 43). The stringency of hybridization was calculated from an assumed visna virus G+C content (51%) and the following relationship (24): $T_m = 81.5 + 16.6 (\log M) + 0.41$ (percent G+C) $- 0.72$ (percent formamide), in which M is the monovalent salt molarity and (percent G+C) is the percentage G+C residues in the DNA. The effective temperatures (which are expressed at $T_m - \Delta T$, in which ΔT is the difference between T_m and the temperature at which the heteroduplex was mounted for microscopy) for 20, 30, 40, 50, and 60% formamide hyperphases were T_m −46°C, T_m −39°C, T_m −31°C, T_m −24°C, and T_m −17°C, respectively. All heteroduplexes were performed with inserts in bacteriophage λ. (A) through (C) Actual heteroduplexes. (a) through (c) Interpretive drawings. (A) T_m −46°C. (B) T_m −31°C. (C) T_m −17°C. The 5′ and 3′ ends of the inserts and the λ arms are indicated. Highly homologous regions in *gag* and *sor* are noted by solid and open arrows, respectively. All micrographs are at the same magnifications.

the 9.4-kb fragment was gel purified, dephosphorylated, and ligated to the gel-purified 0.4-kb fragment. This ligation mixture, when transfected onto goat synovial membrane cells by the calcium phosphate coprecipitation method of Graham and Van der Eb (18), induced syncytium formation. Control cells treated similarly with carrier salmon sperm DNA did not. To determine that infectious virus was also released, the supernatant medium was harvested and placed on fresh goat synovial membrane cells. Once again, syncytia were induced and have provided an infectious stock of CAEV of known genetic composition.

To examine the nucleic acid homology between CAEV and visna virus DNAs, we first used heteroduplex mapping in combination with thermal-melt analyses, whereby the relative stringency of hybridization conditions in spreads is varied by changing the concentration of the denaturing agent (formamide) and monovalent cation in the spreading solution (8, 43). With this technique, duplexed regions of DNA of weak homology (substitutions) can be observed to melt out into single-stranded DNA at increasing stringencies depending on the relative degree of nucleotide sequence conservation between them. Under low-stringency conditions (melting temperature [T_m], −46°C), the genomes appeared to be completely homologous, with the exception of a single substitution (α) which occurred in the amino terminus of the *env* gene in 100% of the molecules (Fig. 2 and 3A). Two minor substitutions, one 5′ (β) and the other 3′ (γ) of the α substitution, were each present in 50% of the molecules. The β substitution appeared to occur in the 3′ terminus of the *sor* gene of visna virus, whereas the γ substitution appeared to reside in about the middle of the *env* gene. The β and γ substitutions increased in frequency to 100% and, along with the α substitution, were the only nonduplexed features present at T_m −39°C. At T_m −31°C, additional small substitutions (ε_1, ε_2, ε_3) occurred in the *gag* and *pol* genes in 39, 50, and 22% of the heteroduplexes, respectively. A new *env* substitution (δ) was present in 22% of the molecules. As the stringency of spreads was increased further (T_m −24°C), additional substitutions occurred in the *gag* and *pol* genes (κ_1, κ_2, and κ_3) and the α, β, and γ substitutions in the *env* and *sor* regions increased in size until they appeared as one large substitution in 50% of the molecules. In the other 50%, a small region of note between the α and β substitutions remained duplexed. At the highest stringency used (T_m

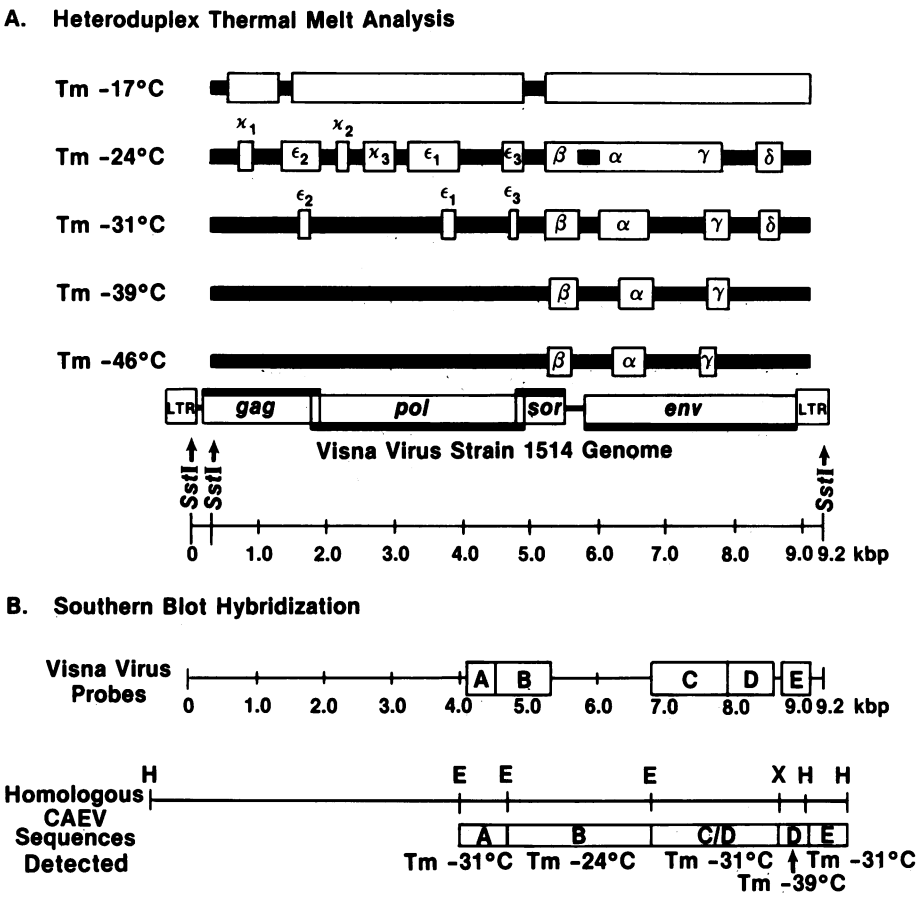

FIG. 3. Sequence homology between molecular clones of CAEV and visna virus strain 1514. (A) Schematic diagram demonstrating genome divergence between clones of visna virus strain 1514 (25) and CAEV (clone 9.4), as determined by heteroduplex thermal melt analysis (Fig. 2). Homologous parts of the genomes (solid bars) and substitutions (open bars) are drawn to scale and are shown in relation to the coding regions of the visna virus genome as determined by nucleotide sequence analysis (40). At least 24 molecules were evaluated at each stringency. Analyses were compiled from five different stringencies, T_m −46°C, T_m −39°C, T_m −31°C, T_m −24°C, and T_m −17°C, which allow for base mismatch up to 32, 27, 22, 16, and 11%, respectively. Abbreviations used are as follows: *sor*, short open reading frame; *gag*, core proteins; *pol*, polymerase; *env*, envelope; and LTR, long terminal repeat. (B) Southern blot hybridization. CAEV restriction endonuclease fragments were generated from clone 9.4 by digestion with *Hin*dIII, *Eco*RI, and *Xho*I. Clone 0.4 was digested with *Hin*dIII to release the insert (Fig. 1). Multiple sets of digests were hybridized with different probes at 43°C in hybridization buffer containing 30, 40, 50, or 60% formamide. Hybridization buffer contains 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) 20 μg of tRNA per ml, 20 μg of sheared denatured calf thymus DNA per ml, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 0.02% polyvinylpyrollidine, 0.02% Ficoll, and 0.02% bovine serum albumin. Transfers were washed extensively in 0.1× SSC containing 0.1% sodium dodecyl sulfate under conditions corresponding to the hybridization conditions used for heteroduplexes (Fig. 2 and 3A legend).

−17°C), only three small regions remained duplexed, two in the *gag* gene and sequences in the carboxy terminus of *pol* and overlapping *sor*.

Previous molecular hybridization studies with CAEV and visna virus DNA revealed extensive homology in the 5′ portions of their genomes, but only a small region of homology in the 3′ end of their genomes (34). In this study, however, the heteroduplex analysis of the two cloned viral genomes indicated that more extensive homology existed. To confirm the heteroduplex data in the 3′ end of the genomes, small cloned probes of visna virus, representing different segments of the genome but encompassing the carboxy terminus of *pol* and extending to the LTR, were used to localize regions of homology in CAEV. Cloned visna virus DNA fragments were subcloned into M13mp10 and M13mp11 vectors. The clones containing the following segments of DNA mapped from the 5′ end of the complete visna proviral genome in reference 25: a *Pst*I-*Bam*HI fragment mapping at 4.65 through 5.1 kb, a *Bam*HI-*Eco*RI fragment mapping at 5.1 through 5.8 kb, a *Hpa*II-*Xba*I fragment mapping at 8.0 through 8.9 kb, a *Xba*I-*Hpa*II fragment mapping at 8.9 through 9.4 kb, and a *Hin*dIII-*Sst*I fragment mapping at 9.7 through 10.15 kb. These fragments have been realigned to the visna virus genome in Fig. 3A on the basis of location in the visna virus sequence (40) of the specific restriction endonuclease sites used to generate their termini. Probe primer (P-L Biochemicals, Inc.) was used to synthesize ^{32}P-labeled probe from single-stranded phage DNA. Hybridization of visna virus probes to molecularly dissected cloned CAEV DNA, electrophoresed on an agarose gel and transferred to nitrocellulose, was performed under stringencies similar to that for heteroduplex analysis (Fig. 2 and 3B). The results were complementary to the heteroduplex data and demonstrated that the most highly conserved region in the 3′ half of the genome begins at the carboxy terminus of *pol* and overlaps with *sor* (compare Fig. 2C and 3A and B);

and the least conserved, based on the visna virus probes selected, corresponds to the δ *env* substitution region (compare Fig. 2B and 3A and B).

The nucleotide sequence of visna virus has recently been determined (40). In its genomic organization it is similar to other retroviruses, having *gag*, *pol*, and *env* structural genes located 5′ to 3′ respectively. However, visna virus is most similar to HTLV-III in having an extra open reading frame (*orf*), called *sor*, located immediately to the 3′ end of the *pol* gene and partially overlapping its carboxy terminus (26, 35, 37, 40, 42). HTLV-III has a second *orf* (3′-*orf*), which overlaps the carboxy terminus of the *env* and extends into the LTR (26, 35, 37, 42); a 3′-*orf* for visna virus has not been defined (40). On the basis of the extensive homology which exists between CAEV and visna virus as seen in the heteroduplexes in this study, the deduced genomic organization for CAEV must be the same as for visna virus. Grossly, the greatest homology exists in the *gag* and *pol* genes, which would be expected to be the most highly conserved, since these genes code for the core and the reverse transcriptase proteins of the viruses and are highly conserved in the evolution of retrovirus groups. From analysis of heteroduplexes at various stringencies, this region contains 78% homologous base pairs.

Surprisingly, two other regions, *sor* and a region between *sor* and the *env* gene, were also highly conserved. The conservation of sequences in the *sor* gene suggests that it codes for a protein that is functionally important for virus replication, although a gene product or subgenomic mRNA for this region has not been identified. The second region between *sor* and *env* in HTLV-III contains a gene called *tat*, for *trans*-activating transcriptional regulation, which encodes a protein that is believed to modulate viral transcription in infected cells (2, 39). An analogous structural region exists in visna virus (40). Cells infected with visna virus also *trans*-activate the U3 regions of either visna virus or CAEV to the same extent; it therefore appears that a *tat* gene also exists for visna virus and is strongly conserved both structurally and functionally between visna virus and CAEV (21).

Molecular hybridization and heteroduplex mapping revealed that the most divergent genetic regions between CAEV and visna virus clones exist in the 3′ *env* gene sequences. This region encodes the glycosylated exterior membrane and the transmembrane proteins (40). The most variable regions, α and γ (Fig. 3A), occurred in the 5′ half of the *env* gene, which is the region which encodes the exterior envelope glycoprotein; the other region, δ (Fig. 3A), appeared to map in the transmembrane region. A similar distribution of substitutions are present in divergent HTLV-III clones, with the amino-terminal *env* gene substitution being the most divergent, as demonstrated by heteroduplex mapping (20). The variable domains in the envelope glycoprotein are of particular interest, since they may be the regions which encode the epitopes involved in tissue specificity and neutralization.

CAEV and visna virus are closely related lentiviruses of goats and sheep, respectively, which cause the same multiorgan diseases. However, there are marked differences in their cell tropism in cells in culture, their neurotropic potential in their respective hosts, and the immunologic response of naturally infected hosts to these two viruses. Sheep produce neutralizing antibodies in response to visna virus infection, but virus persists in spite of this strong immune response. Envelope variants that escape immune surveillance mechanisms arise in the infected animal (antigenic drift) (3, 27, 28). CAEV, in contrast, does not induce neutralizing antibodies, although antibodies against the envelope glycoprotein are present (22, 30). Thus, the strategies for developing effective vaccines to these viruses may be complicated. The availability of biologically active molecular clones for analyses and genetic engineering may be helpful in this respect. The CAEV clones reported here are the first infectious lentivirus clones to be isolated.

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