Genetic Variation Among Lentiviruses: Homology Between Visna Virus and Caprine Arthritis-Encephalitis Virus Is Confined to the 5' *gag-pol* Region and a Small Portion of the *env* Gene

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Visna virus of sheep and arthritis-encephalitis virus of goats are serologically related but genetically distinct retroviruses which cause slowly progressive diseases in their natural hosts. To localize homologous regions of the DNAs of these two viruses, we constructed a physical map of caprine arthritis-encephalitis virus DNA and aligned it with the viral RNA. Cloned probes of visna virus DNA were then used to localize regions of homology with the caprine arthritis-encephalitis virus DNA. These studies showed homology in the 5' region of the genome encompassing U5 and the *gag* and *pol* genes and also in a small region in the *env* gene. These findings correlate with biological data suggesting that the regions of the DNA which are homologous may be responsible for virus group characteristics such as the closely related virus core antigens. Regions which did not show homology such as large sections in the *env* gene may represent unique sequences which control highly strain-specific characteristics such as the neutralization antigen and specific cell tropisms.

Lentiviruses of sheep and goats are a distinct group of nononcogenic retroviruses which cause slowly progressive diseases involving the central nervous system, lungs, and joints (8, 9, 12, 13, 17, 35, 43). The lesions are characterized by persistent inflammation (active-chronic type) which eventually leads to paralysis, respiratory failure, and crippling arthritis (8, 9, 10, 11). Sheep develop mainly the pneumonic form of the disease complex, and this leads to neurological complications in some animals (17, 35). In goats, the disease expression is age dependent with neurological disease occurring mainly in young animals and arthritis occurring mainly in adults (8, 9, 10, 11). The sheep viruses, of which visna is the prototype, were discovered during epizootics of pneumoencephalitis (maedi-visna) in Iceland in the 1940s (40, 41), and the caprine viruses were discovered during an outbreak of arthritis and encephalitis among goats in Washington state in the 1970s (8, 10). These viruses are widespread in sheep and goat populations in many parts of the world. In experiments, both viruses cause persistent infections and replicate at a slow but continuous rate for an indefinite period. The continuous release of viral antigens in specific tissues stimulates the inflammatory response which leads to disease.

Visna virus and caprine arthritis-encephalitis virus (CAEV) have different mechanisms for maintaining persistent replication in their immunocompetent hosts. Both viruses induce binding antibodies to all their respective polypeptides during infection. However, they differ greatly with respect to induction of neutralizing antibodies. CAEVs do not induce neutralizing antibodies during natural or experimental infections, in contrast to visna viruses, which do so readily (24, 33). Neutralizing antibodies to visna viruses are highly specific. However, the range of neutralization is too narrow to inhibit replication of mutant viruses which arise in the immune host. The result of this narrow neutralization range is selective replication of virus mutants in the persistently infected animal (antigenic drift) (4, 30, 31). These two viruses also vary in their efficiency of replication in sheep fibroblasts. Visna viruses cause a highly productive lytic infection in these cultures. In contrast, CAEVs cause a

noncytopathic persistent infection in which virus replication is restricted more than 1,000-fold (34).

To determine the genetic basis for the differences in host responses to these two viruses, we have begun studies on the nucleic acids of prototypes of these two virus groups, focusing initially on regions of homology between their DNAs. Previous hybridization studies have shown that there is only minor homology between the nucleic acids of visna virus and CAEV (16, 38). However, the physical location of homologous and nonhomologous regions between the two viral genomes has not been determined. In this study, we constructed a restriction endonuclease map of the DNA of CAEV and aligned it with the viral RNA. With cloned fragments of visna virus DNA (28) and different degrees of stringency for hybridization, we localized regions of nucleic acid homology and identified regions of the genomes of the two viruses which were distinct. These genetic differences and similarities were then compared with the immunological specificities of viral polypeptides determined in immunoprecipitation tests and virus neutralization experiments. These studies provide new insights into the relationship between viral genes, gene products, and antiviral host responses and suggest an experimental basis for evaluating the role of viral genes in the outcome of these slow infections.

MATERIALS AND METHODS

Virus and cells. CAEV strain CO was used in this study. The history and characterization of the agent have been reported previously (8, 29, 33). The virus was plaque purified and propagated in cultures of goat synovial membrane (GSM) cells as described earlier (29). Visna virus strain 1514 was used in this study; the plaque-purified virus stock was grown in sheep choroid plexus cells (SCP) as previously described (30, 32).

DNA. GSM or SCP cells were infected at a multiplicity of infection of 0.5 to 1 and harvested at peak cytopathic effect (5 to 7 days postinfection). Low-molecular-weight DNA was isolated as described by Hirt (19), and the supernatant DNA was processed as described by Clements et al. (7) except that the RNase A digestion was for 30 min, and digestion with 50

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µg of proteinase K per ml–0.5% sodium dodecyl sulfate was substituted for the pronase digestion.

Enzymes. Restriction enzymes were purchased from Bethesda Research Laboratories, Rockville, Md., or New England BioLabs, Beverly, Mass., and were used under conditions suggested by Maniatis et al. (27). Other purchased enzymes were reverse transcriptase (Life Sciences, St. Petersburg, Fla.), RNase-A (Calbiochem-Behring, La Jolla, Calif.), and proteinase K (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.).

Virus purification. Cells (either GSM cells for CAEV or SCP cells for visna virus) were infected at a multiplicity of infection of 0.5 to 1 in 850-cm² roller bottles (Corning Glass Works, Corning, N.Y.), and supernatants from the infected cultures were collected 1 to 2 times per day for 5 to 6 days. After clarification of the supernatant fluid at 8,000 rpm in a GSA rotor (Ivan Sorvall, Inc., Norwalk, Conn.) for 30 min, the virus was concentrated (100-fold) with a pellicon membrane concentrator (Millipore Corp., Bedford, Mass.) and then pelleted at 27,000 rpm in an AH627 rotor (Sorvall) at 4°C. The virus was suspended in 0.1 M NaCl–1 mM EDTA–20 mM Tris (pH 7.4), diluted 1:1 with 5.3 M NaCl–1 mM EDTA–20 mM Tris (pH 7.4) (45), incubated at 37°C for 30 min, and diluted 1:1 with 20 mM Tris (pH 7.4)–1 mM EDTA. Virus was banded by centrifugation for 2 h at 20,000 rpm in a Sorvall SS-90 at 4°C on a step gradient of 55 to 20% sucrose in 1 M NaCl–1 mM EDTA–20 mM Tris (pH 7.4). Virus was pelleted at 27,000 rpm in an AH627 rotor (Sorvall) and suspended in 0.1 M NaCl–1 mM EDTA–20 mM Tris (pH 7.4) (2,000-fold concentration of original supernatant).

Preparation of viral RNA. Virion RNA was released by lysis of the virions with 200 µg of proteinase K per ml–1% SDS at 37°C for 2 h. The RNA was centrifuged through a 15 to 30% sucrose gradient containing 0.1 M NaCl–20 mM Tris (pH 7.4)–1 mM EDTA–0.2% SDS in a Beckman SW41 rotor for 2 h at 38,000 rpm and 22°C. Optical density at 254 nm was monitored with an ISCO model 328 fraction collector, and the peak fractions were pooled. The pooled fractions were adjusted to 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and loaded onto an oligodeoxythymidylic acid column (Collaborative Research, Inc., Waltham, Mass.). Polyadenylated [poly(A)⁺] RNA was eluted with 10 mM Tris (pH 7.4) and was ethanol precipitated.

Hybridization probes. Representative ³²P-labeled CAEV and visna virus 1514 cDNA probes were prepared from virion RNA (44). CAEV and visna virus 1514 ³²P-labeled “strong-stop” DNA specific for the 5′ end of the virion RNA was synthesized as previously described (18), denatured by heating at 90°C for 2 min in 50% formamide, and purified on a 10% polyacrylamide gel with 90 mM Tris-borate (pH 8.3)–1 mM EDTA at 400 V for 3 to 4 h. The strong-stop band was eluted from the gel into 100 µg of tRNA per ml–5 mM EDTA. The eluted band was treated overnight with 0.24 N NaOH at 37°C to hydrolyze the complementary RNA and then purified on a second polyacrylamide gel. The strong-stop band was eluted as described above and used in hybridization studies described below. A 3′-specific probe was synthesized with fragmented poly(A⁺) virion RNA as described in a previous report (28).

*Sst*I clone 8.5 of visna virus 1514 (28) was digested with *Pst*I and *Sst*I, and the resulting fragments were individually subcloned into the *Pst*I site of pBR322. These subclones map at 1.6 to 2.9 kilobases (kb) (B), 2.9 to 4.65 kb (C), 6.2 to 6.8 kb (D), and 6.9 to 8.8 kb (E) from the 5′ end of visna virus DNA (5). These subclones were nick translated to high specific activity (5.3 to 10⁷ to 1.2 × 10⁸ cpm/µg) (27, 37).

Two probes for the U3 region were subcloned into M13mp10 from the *Sst*I clone 8.5 and included 8.9 to 9.7 kb (F) and 9.7 to 10.15 kb (G) from the 5′ end of the visna virus DNA. A 5′ fragment (0.9 to 1.95 kb from the 5′ end of the visna virus DNA) from the *Sst*I 8.5 clone of strain 1514 was also subcloned into M13mp10 (A). These subclones were labeled by using the probe primer (P-L Biochemicals, Inc., Milwaukee, Wisc.) and the conditions described by Ricca et al. (36). The specific activities of these probes ranged from 7.4 × 10⁶ to 1.6 × 10⁷ cpm/µg.

Agarose gel electrophoresis. DNA was analyzed by electrophoresis in 1.2% 3-mm agarose gels, transferred to nitrocellulose by the method of Southern (42), and hybridized to different probes under conditions described below.

Hybridization of probes to DNA transfers. For mapping studies, DNA transfers were hybridized in 50% formamide (FLUKA AG) containing 6× SSC, 20 µg of single-stranded DNA per ml, 20 µg of tRNA per ml, 50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (pH 7), 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin at 42°C for 36 to 48 h. DNA transfers were washed for 15 min in 2× SSC at 23°C, 1 h in 1× SSC–0.1% SDS at 50°C, and 2 min in 1× SSC at 23°C. DNA transfers were air dried and exposed to Kodak XAR-5 film at –70°C with intensifying screens.

Hybridization conditions used in homology studies were similar to those described by Howley et al. (20). DNA transfers were hybridized as for mapping except that the formamide concentration was varied, and hybridization was performed at 35°C. Preliminary washes were in 2× SSC for 30 min at 23°C. The washing temperatures used were calculated by the following formula: $T_m = 81.5 + 16.6(\log M) + 0.41(\%G+C) - 0.72\%$ formamide where T_m is the melting temperature and M is the molarity of monovalent salts (20). For visna virus DNA, %G+C equals 51% (25). The extensive washes in 1× SSC–0.1% SDS were at temperatures equivalent to the stringencies of the 35°C hybridization conditions with different formamide concentrations; specifically, the temperatures of the washes were as follows: for 20% formamide, 36°C; for 30% formamide, 44°C; for 40% formamide, 51°C; and for 50% formamide, 58°C. DNA transfers were rinsed briefly in 1× SSC, dried, and exposed to film.

Purification of visna virus glycoprotein. The envelope glycoprotein gp135 was purified by affinity chromatography on a lens culinaris Sepharose 4B column (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). Banded visna virus (10 mg of total viral protein in 10 ml) was disrupted in a buffer containing 1% Zwittergent SB-14, 0.1% Triton X-100, 0.15 M NaCl, 0.02 M Tris (pH 7.4), and 60 mM dithiothreitol. The disrupted virus was loaded on the column (3.5 ml), washed with 30 column volumes of wash buffer (0.1% Zwittergent SB-14 in 0.15 M NaCl and 0.02 M Tris [pH 7.4]). The glycoprotein was eluted in 1 to 2 column volumes by 0.2 M α-methylmannoside in the wash buffer. [³H]leucine-labeled virus was added to the banded virus to monitor protein recovery; 5 to 8% of the total viral protein was recovered from the column. Analysis of the purified protein by SDS polyacrylamide gel electrophoresis revealed a single major protein band which migrated with a molecular weight of 135,000.

Purification of visna virus core protein. The core protein p27 of visna virus was purified from banded virus (3 mg) and ³⁵S-labeled virus on Bio-Gel A-5M (Bio-Rad Laboratories, Richmond, Calif.) by using 6 M guanidine HCl. Virus was disrupted in 8 M guanidine HCl–50 mM Tris (pH 8.5)–10 mM EDTA–2% 2-mercaptoethanol. Disrupted virus was applied

FIG. 1. Restriction endonuclease analysis of CAEV DNA. (A and B) CAEV DNA was subjected to electrophoresis in 1.2% agarose gels, transferred to nitrocellulose filters, and hybridized with representative probe (see text). Lanes in (A) contain CAEV DNA (unless otherwise indicated) treated as follows: a, undigested; b, digested with *XhoI*; c, digested with *XhoI* and *EcoRI*; d, digested with *EcoRI*; e, digested with *BstEII* and *EcoRI*; f, digested with *BstEII*; g, digested with *SmaI* and *EcoRI*; h, digested with *SmaI*. Lanes in (B) contain CAEV DNA treated as follows: i, undigested; j, partial digestion with *EcoRI*; k, complete digestion with *EcoRI*. The sizes in kb of *HpaI* fragments of λ DNA are indicated on the left of (A) and (B). The calculated sizes (in kb) of partial *EcoRI* fragments are indicated on the right of (B). (C) Interpretation of *EcoRI* partial-digestion products.

to a column (90 by 1.5 cm) of Bio-Gel A-5M which had been equilibrated with 6 M guanidine HCl–0.02 M NaHPO₄ (pH 6.5)–10 mM dithiothreitol. After elution of proteins, samples of each fraction were counted. The peak for p27 was pooled and analyzed by SDS-polyacrylamide gel electrophoresis. A single major protein band which migrated with a molecular weight of 27,000 was observed.

Preparation of antibody to visna virus and CAEV proteins. Antibody to the visna virus glycoprotein was prepared in guinea pigs by using 40 μ g of gp135 emulsified in Freund complete adjuvant. This material was injected intradermally in four sites with 0.5 ml injected per site. At 3 and 5 weeks after inoculation, the animals were boosted with 40 μ g of gp135 given intraperitoneally. The animals were bled for serum 1 week after the last injection. The guinea pig anti-gp135 sera are monospecific and immunoprecipitated only the gp135 from virus. The sera also contain neutralizing antibodies to visna virus.

Antibody to the visna virus core protein p27 was prepared by injecting multiple inoculations of 20 μ g of p27 in Freund complete adjuvant into a goat. The goat anti-p27 sera is monospecific and immunoprecipitated only the p27 protein of visna virus.

Antibody to CAEV was prepared by several injections of UV-inactivated virus into a goat (g40) (33). The initial inoculum consisted of virus emulsified in Freund complete adjuvant, which was followed by virus in incomplete adjuvant. Antigen was subsequently given intravenously, and the immunization schedule ended with a final injection of infectious virus.

Immunoprecipitation of viral proteins. Visna virus and CAEV, at a multiplicity of infection of 1, were used to infect SCP and GSM cells, respectively, in 150-cm² flasks. When the viral cytopathic effect was maximal, cells in each flask were labeled with 5 ml of methionine-free minimal essential medium supplemented with 50 μ Ci of [³⁵S]methionine per ml (600 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), 5% normal minimal essential medium and 0.5% fetal calf serum. The flasks were incubated at 37°C for 12 h after which the medium was removed and replaced with 5 ml of fresh medium with label. This process was repeated twice, the supernatants from the three 12-h periods were pooled and clarified, and the virus was pelleted as described above. The virus was lysed in a buffer containing 0.5% Zwittergent SB-14, 0.5% deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.02 M Tris (pH 7.6), and 1 mM phenylmethylsulfonyl fluoride. Antibody and virus (200,000 cpm) were incubated at 37°C for 2 h, and rabbit anti-goat antibody (40 μ l; Miles Laboratories, Inc., Elkhart, Ind.) was added when goat sera were used, and this mixture was incubated for 1 h at 37°C. Protein A-Sepharose beads were added and incubated overnight at 4°C. The beads were washed twice with lysing buffer and twice with 0.5 M LiCl–0.5% Nonidet P-40–0.5% Zwittergent SB-14–0.025 M Tris (pH 7.6). The proteins were removed from the beads by boiling in 1% SDS–0.1 M 2-mercaptoethanol and were analyzed by electrophoresis in a 5 to 20% acrylamide gel containing 0.1% SDS. The gels were fluorographed (2) and exposed to X-ray film at –70°C.

RESULTS

Construction of the CAEV restriction map. Hirt supernatant DNA was prepared from infected GSM cells. This DNA was digested with restriction endonucleases, transferred to nitrocellulose (DNA transfers), and hybridized with a representative cDNA probe to determine the location of restriction endonuclease cleavage sites on the viral DNA. The results of initial restriction enzyme digests were consistent with the majority of the viral DNA being a double-stranded linear molecule of 10.3 kb. Partial and complete digestions with *EcoRI* suggested a probable ordering of the fragments (Fig. 1). The one-cut enzymes *BglII*, *BstEII*, *SmaI*, and *XhoI* were used in double digests to further define the map (Fig. 1 and 2). Restriction sites have been mapped for the enzymes *BglI*, *BglII*, *BstEII*, *EcoRI*, *HindIII*, *KpnI*, *PstI*, *SmaI*, *SstI*, *XbaI*, and *XhoI* (Fig. 2). No sites were detected for the enzymes *BclI*, *Clal*, *Sall*, or *XorII*.

Orientation of the CAEV DNA with respect to the genomic RNA. To orient the linear viral DNA map with the genomic RNA, 5'- and 3'-specific probes were synthesized from the virion RNA. The 146-base strong-stop probe contains U5 and R sequences from the 5' end of the genomic RNA and, by analogy with other retroviruses, would be expected to hybridize to the long terminal repeat structure at both ends of the linear DNA.

Duplicate sets of single and double *SstI*, *HindIII*, and *KpnI* digestions of Hirt supernatant DNA were subjected to agarose gel electrophoresis and then transferred to nitrocellulose by the method of Southern (42). One set was hybridized with the representative cDNA probe, and the other set

FIG. 2. A physical map of CAEV DNA with the cleavage sites of restriction enzymes indicated. The total length of the genome is 10.3 kb. Abbreviations and locations of cleavage sites (in kb) from the 5' end of the genome are as follows: B, *BglII* (7.6, 8.2, 9.0); Bg, *BglII* (3.1); Bs, *BstEII* (4.6); E, *EcoRI* (4.5, 5.2, 7.1); H, *HindIII* (0.2, 9.6, 10.0); K, *KpnI* (0.3, 7.0, 10.1); P, *PstI* (5.0, 6.1); Sm, *SmaI* (6.7); Ss, *SstI* (0.8, 1.3); Xb, *XbaI* (0.8, 4.95); and Xh, *XhoI* (9.2). Arrows, three restriction sites common to CAEV and visna virus DNA as follows: Ss, 0.9 kb on the visna virus 1514 map and 0.8 kb on the CAEV map; P, 6.2 kb on the visna virus 1514 map and 6.1 kb on the CAEV map; and H, 9.7 kb on the visna virus 1514 map and 9.6 kb on the CAEV map.

was hybridized with the strong-stop probe (Fig. 3). The *KpnI* digest was especially useful for determining the orientation of the CAEV DNA with respect to the genomic RNA (Fig. 3). This enzyme cuts the DNA into two major internal

fragments of 6.7 and 3.1 kb and also cuts within each long terminal repeat. Strong-stop probe hybridized with the 6.7-kb fragment but not with the 3.1-kb fragment. This pattern is consistent with a cleavage site near or at the boundary between U3 and R. The 6.7-kb fragment can be assigned to the 5' end since it contains R and U5 and hybridizes strongly with the strong-stop probe. Similar analysis of the patterns of hybridization of strong-stop probe with the other restriction digests shown in Fig. 3 confirmed this orientation of the DNA restriction map.

Analysis of the pattern of hybridization of the 3' probe with CAEV DNA digested with *KpnI* showed very strong hybridization with the 3.1-kb fragment and much reduced hybridization to the 6.7-kb fragment (Fig. 4). Minor hybridization with the 6.7-kb fragment is thought to be due to the large size of the 3' probe. Hybridization of the 3' probe with CAEV DNA digested with *PstI* showed hybridization to both the 5' and 3' fragments, as would be expected because of repeated sequences at the ends of the genome. After double digestion with *KpnI* and *PstI*, strong hybridization was detected only to the 3.1-kb *KpnI* fragment. Similarly, when CAEV DNA was digested with either *EcoRI* and *XbaI* or *BglII*, *XbaI*, and *XhoI*, the 3' probe hybridized only to one

FIG. 3. Hybridization of restriction endonuclease-digested CAEV DNA with 32 P-labeled CAEV strong-stop probe. CAEV DNA was subjected to electrophoresis in a 1.2% agarose gel, transferred to nitrocellulose, and hybridized with either CAEV-representative probe (A) or CAEV strong-stop probe (B). The lanes contain CAEV DNA treated as follows: a, undigested; b, digested with *SstI*; c, digested with *SstI* and *HindIII*; d, digested with *HindIII*; e, digested with *HindIII* and *KpnI*; f, digested with *SstI* and *KpnI*; g, digested with *KpnI*. Sizes in kb and locations in the gel of *HpaI* fragments of λ DNA are indicated on the left. (C) Map for each digest is indicated: open boxes, fragments hybridizing with CAEV strong-stop probe.

FIG. 4. Hybridization of restriction endonuclease-digested CAEV DNA with ^{32}P -labeled probe enriched for sequences at the 3' end of CAEV RNA (3' probe). CAEV DNA was subjected to electrophoresis in a 1.2% agarose gel, transferred to nitrocellulose, and hybridized with either representative probe (A) or with 3' probe (B). Lanes contain CAEV DNA (unless otherwise indicated) treated as follows: a, undigested; b, digested with *Pst*I; c, digested with *Pst*I and *Kpn*I; d, digested with *Kpn*I; e, digested with *Eco*RI and *Xba*I; f, digested with *Eco*RI; g, digested with *Bgl*II, *Xba*I, and *Xho*I. Sizes in kb and locations in gel of *Hind*III fragments of λ DNA are indicated on the left. (C) Restriction endonuclease map for each digest is indicated. Open boxes, fragments hybridizing strongly with the 3' probe.

terminal fragment (Fig. 4). This consistent pattern of hybridization of the 3' probe with fragments generated from one end of the viral DNA defined this as the 3' end with respect to the virion RNA.

Common restriction endonuclease sites in the DNAs of CAEV and visna virus. Examination of the restriction enzyme maps of the DNAs of visna virus (5) and CAEV revealed common sites between the DNAs. These common sites are indicated by arrows in Fig. 2.

Nucleic acid homology between CAEV and visna virus DNA. To localize any homologous regions shared by CAEV and visna virus genomes, CAEV DNA was digested with the restriction enzyme *Eco*RI, transferred to nitrocellulose, and hybridized with either CAEV or visna virus-representative cDNA probes under four different hybridization stringencies (see above). The lower panel of Fig. 5 shows that the CAEV-representative probe hybridized to the appropriate fragments under all hybridization and washing conditions. The upper panel shows decreasing hybridization of visna virus cDNA probes to certain *Eco*RI fragments of CAEV DNA under increasingly stringent conditions. All bands were detectable (some faintly) at 30% formamide. Since every 1% base mismatch lowers the T_m by 1.4°C (23), one can calcu-

late from the hybridization conditions (30% formamide) used that there can be an average of 33% mismatch. At 50% formamide, only the most 5' *Eco*RI fragment hybridized; this hybridization condition allows an average of 22% base mismatch in the fragment. A similar experiment with a triple digestion (*Bgl*II-*Xba*I-*Xho*I) of CAEV DNA confirmed this pattern of strong hybridization to the 5' region of the genome.

Small cloned probes of visna virus described above were used to further localize regions of homology (Fig. 6) and to determine whether small probes would reveal additional nucleic acid homology between the two viral CAEV DNA fragments generated by single digestion with *Eco*RI and triple digestion with *Bgl*II-*Xba*I-*Xho*I (Fig. 6). Probes A (0.9 to 1.95 kb) and B (1.6 to 2.9 kb) hybridized to the *Eco*RI fragment mapping at 0 to 4.5 kb from the 5' end of the CAEV DNA and to the *Bgl*II-*Xba*I-*Xho*I fragment mapping at 0.8 to 3.1 kb from the 5' end of the CAEV DNA. Probe C (2.9 to 4.65 kb) hybridized to the *Eco*RI fragments mapping at 0 to 4.5 and 4.5 to 5.2 kb from the 5' end of the CAEV DNA and to the *Bgl*II-*Xba*I-*Xho*I fragments mapping at 0.8 to 3.1 and 3.1 to 4.95 kb from the 5' end of the CAEV DNA. Probe E (6.9 to 8.8 kb) hybridized to the *Eco*RI fragment mapping at

FIG. 5. Hybridization of CAEV DNA with representative ³²P-labeled CAEV cDNA or representative ³²P-labeled visna virus cDNA at different stringencies. DNA was subjected to electrophoresis in a 1.2% agarose gel and transferred to nitrocellulose. DNA transfers were hybridized at 35°C with 20, 30, 40 or 50% formamide in the hybridization buffer and were washed in 1× SSC–0.1% SDS at temperatures equivalent to the hybridization conditions (see text). Sizes in kb and locations in the gel of *Hpa*I fragments of λ DNA are indicated on the right of each panel of the figure. Arrows, the four bands generated by digestion of CAEV DNA with *Eco*RI. (A) Hybridization with ³²P-labeled visna virus cDNA. Lanes a to d contain CAEV DNA digested with *Eco*RI and hybridized under the following conditions: a, 20% formamide and a 36°C wash; b, 30% formamide and a 44°C wash; c, 40% formamide and a 51°C wash; and d, 50% formamide and a 58°C wash. Lane e, Visna virus DNA digested with *Bam*HI, hybridized with 50% formamide, and washed at 58°C; f, undigested visna virus DNA hybridized with 50% formamide and washed at 58°C. (B) Hybridization with ³²P-labeled CAEV cDNA. Lanes g to j contain CAEV DNA digested with *Eco*RI and hybridized under the following conditions: g, 20% formamide and a 36°C wash; h, 30% formamide and a 44°C wash; i, 40% formamide and a 51°C wash; and j, 50% formamide and a 58°C wash. Lane k, Undigested CAEV DNA hybridized with 50% formamide and washed at 58°C.

7.1 to 10.3 kb from the 5' end of the CAEV DNA and to the *Bgl*II-*Xba*I-*Xho*I fragment mapping at 4.95 to 9.2 kb from the 5' end of the CAEV DNA. The visna virus probes from 6.2 to 6.8 kb (probe D) and 8.9 to 10.15 kb (probes F and G) from the 5' end of the genome did not hybridize to the CAEV DNA fragments generated by single or triple digests. Cloned probe for the visna virus U5 or strong-stop region was not available. To measure cross-homology to this region, strong-stop visna virus DNA was synthesized (18) and hybridized to the *Eco*RI fragments mapping at 0 to 4.5 and 7.1 to 10.3 kb from the 5' end of the CAEV DNA.

Antigenic relationship of the proteins of CAEV and visna virus. Immunoprecipitations of viral proteins with different

sera were performed to determine whether different proteins encoded by the homologous regions of the viral genomes shared antigenic determinants (Fig. 7). ³⁵S-labeled proteins of CAEV and visna virus were reacted with antisera prepared against proteins of each virus as follows: a hyperimmune goat serum against CAEV (g40 serum), guinea pig serum against visna virus glycoprotein, and a goat serum against visna virus core protein. The glycoproteins of both CAEV and visna virus were immunoprecipitated by the anti-CAEV serum and by the guinea pig antisera to the glycoprotein of visna virus. Similarly, the core proteins of both CAEV and visna virus were precipitated with the visna virus anti-core protein serum.

FIG. 6. (I) Hybridization of seven subcloned visna virus probes and visna virus strong-stop probe (see text) with single (*Eco*RI) or triple (*Bgl*II-*Xba*I-*Xho*I) digests of CAEV DNA subjected to electrophoresis in 1.2% agarose gels and transferred to nitrocellulose. The restriction enzyme cleavage sites for the *Eco*RI digest and the *Bgl*II-*Xba*I-*Xho*I triple digest are shown at the bottom of the figure. Open boxes, CAEV DNA fragments hybridizing with each probe on the restriction map for each digest; bars, locations of the probes on the visna virus restriction map. The sites of *Eco*RI cleavage are shown above the lines, and the sites of *Bgl*II-*Xba*I-*Xho*I cleavage are shown below the lines. Strong-stop probe was hybridized with only the *Eco*RI digest. Locations of the probes (in kb) from the 5' end of the visna virus map are as follows: strong-stop probe (SS), 0.35 to 0.5 and 10.15 to 10.3; A, 0.9 to 1.95; B, 1.6 to 2.9; C, 2.9 to 4.65; D, 6.2 to 6.8; E, 6.9 to 8.8; F, 8.9 to 9.7; and G, 9.7 to 10.15. No hybridization of restriction fragments was detected with probes D, F, and G. (II) A. Genetic organization of visna virus. B. Summary of regions of homology between CAEV and visna virus.

DISCUSSION

In this report, regions of homology between the DNAs of two biologically distinct ruminant lentiviruses, visna virus of sheep and arthritis-encephalitis virus of goats, were determined. A restriction enzyme map of CAEV DNA was constructed to align the homologous and nonhomologous regions with the map of visna virus DNA. Experiments with varied stringencies of hybridization of visna virus-representative cDNA probe with CAEV DNA transfers indicated stronger homology at the 5' end of the genome than over the rest of the genome. The experiments with cloned probes showed that there was extensive homology at the 5' end of the genomes and also in a region of the 3' end of the two viral genomes. This latter region corresponds to the 3' end of the *env* gene in visna virus. However, the genetic organization of CAEV is not yet known, although it would be expected that the 5' region contains the *gag* and *pol* genes and that the

3' region contains the *env* gene as established for other retroviruses. These results correlate well with studies of avian retroviruses (21, 22) and feline leukemia viruses (15), which showed that the 3' half of the *env* gene is conserved, whereas the 5' half of the gene diverges. These regions of homology may therefore be common among closely related retroviruses.

In contrast to our studies, other investigations have found only 20 to 30% homology between visna virus DNA and CAEV DNA (16, 38). The hybridization methods (RNA:DNA) and degrees of stringency used in these reports were very different from those reported here. The low-stringency conditions used in our study included an equivalent hybridization temperature which was 52.2°C below the T_m , and a high-stringency temperature which was 30.6°C below the T_m . Conditions used by Roberson et al. (38) for RNA:DNA hybridization were more stringent than our highest stringency, and they detected only 20% homology.

FIG. 7. Immunoprecipitation of CAEV and visna virus proteins with different sera. Lanes a, ^{35}S -labeled CAEV; b, ^{35}S -labeled visna virus; c, g, i, k, and l, CAEV; d, h, and j, visna virus; e, ^{35}S -labeled GSM cells infected with CAEV; f, ^{35}S -labeled SCP cells infected with visna virus. The labeled virus and cell lysates were immunoprecipitated with the following sera: anti-CAEV serum, lanes c, d, e, and f; anti-visna virus p27 serum, lanes g and h; anti-visna virus gp135 serum, lanes i and j; preimmune guinea pig serum, lane k; and preimmune goat serum, lane l. Lane m, Molecular weights ($\times 10^3$) of standard proteins. The left margin indicates the location of the glycoprotein gp135, the major core protein p27, and small core proteins p16 and p15.

The conditions used in the hybridization study by Gazit et al. (16) were more similar to our high-stringency hybridization conditions, and they detected 30% homology. Our study confirms and extends these results by localizing the regions of homology under stringent hybridization conditions and identifying additional regions of homology under less stringent hybridization conditions. In particular, the small cloned probes used in this study allowed a detailed homology map to be constructed and led to the identification of a region of homology in the 3' termini not previously recognized, in addition to the region of homology at the 5' termini of the genomes.

The homology detected in specific regions of the DNA of the two viruses was in general agreement with the immunological relationships of the viral polypeptides. Thus, the homology at the 5' ends of the two genomes was in concordance with the strong antigenic cross-reactivity of the core proteins of the two viruses (1, 6, 29). Detection of homology in the *env* gene also correlated with antigenic cross-reactivity of the glycoproteins as shown in the immunoprecipitation experiments. Immunological cross-reactivity of the glycoproteins was weaker than that of the core proteins but nevertheless was consistent since the antisera to CAEV recognized the glycoprotein of visna virus. In addition, antibody produced against the purified glycoprotein gp135 of visna virus immunoprecipitated the gp135 of CAEV. Further confirmation of the antigenic cross-reaction between the glycoproteins of CAEV and visna virus has been obtained in an enzyme-linked immunosorbent assay with monoclonal antibodies against the gp135 of visna virus (Stanley, Bhaduri, and Clements, unpublished data).

Whereas the concordance between regions of homology and antigenic reactivity of proteins allowed direct extrapolation between genes and gene products, the lack of homology in the DNA provided grounds for speculation on the antigenic diversity of gene products. Previous studies have established that the neutralization antigen(s) of visna virus resided in the glycoprotein (39). Since neutralizing antibodies to visna virus do not neutralize CAEV and vice versa (33) and since titers of preexisting neutralizing antibody to visna virus

in sheep cannot be boosted by injections of CAEV (Narayan, unpublished data), it is probable that the portion of the viral DNA coding for this protein resides in a nonhomologous portion of the *env* gene.

The envelope gene codes for another protein of interest, the p15(E) protein. Sequences which code for this protein in murine leukemia virus are located at the 3' end of the *env* gene. Tissue tropism and disease in murine retroviruses have been linked to this protein and sequences in the U3 region (3, 14, 26). We have recently identified in visna virus a p15(E) protein, but we do not know as yet whether it determines tissue tropism for this virus. If this protein is involved in determining tissue tropism in the two viruses, the gene coding for this protein would not be expected to be homologous because of differences in replication of the two viruses in fibroblastic cultures.

In summary, the regions of homology between the two viruses probably identify viral genes which are conserved among this unique group of retroviruses and control expression of the group phenotype-group virus antigens etc. On the other hand the genomic regions lacking homology, especially those in the *env* gene, may control expression of biological functions such as neutralization and tissue tropism which are unique to each virus strain. Having identified these regions, we hope in future studies to obtain recombinant viruses which will allow further analysis of the biological functions of the viral genes of CAEV and visna virus. This approach may lead to a better understanding of the specific roles viral genes have in the mechanisms of persistent infection and in the pathogenesis of the disease.

ACKNOWLEDGMENTS

We thank Linda Kelly for preparation of the manuscript. We thank Karen Beemon for valuable discussions of the work.

This work was supported by grant RG-1232 from the National Multiple Sclerosis Society and by Public Health Service grants NS 16145 and PO1-NS-15721 from the National Institutes of Health.

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