Nucleotide Sequence and Transcriptional Activity of the Caprine Arthritis-Encephalitis Virus Long Terminal Repeat

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Caprine arthritis-encephalitis virus (CAEV) and visna virus are pathogenic lentiviruses of goats and sheep which share morphologic features and sequence homology with human T-cell lymphotropic virus type III (HTLV-III), the etiologic agent of the acquired immune deficiency syndrome. The nucleotide sequence of the CAEV long terminal repeat (LTR) was determined, and it was found to be 450 base pairs long, with U3, R, and U5 regions of 287, 85, and 78 base pairs, respectively. Portions of the CAEV LTR are closely homologous to analogous regions of visna virus. The CAEV LTR is not significantly homologous with the HTLV-III LTR; however, like HTLV-III, visna virus, and equine infectious anemia virus, CAEV uses tRNA lysine as a primer for reverse transcription. The transcriptional activity of the CAEV and visna virus LTRs was measured by a chloramphenicol acetyltransferase assay, and the activity of the visna virus LTR was generally higher in a variety of uninfected cell types. Infection of cells with visna virus markedly increased gene expression directed by either the CAEV or visna virus LTR, but in contrast, infection of cells with CAEV had little effect on the activity of either LTR. The lack of *trans*-activation by CAEV, a virus which causes debilitating arthritis and encephalitis in goats, suggests that *trans*-activation may not be a general property of pathogenic lentiviruses.

Lentiviruses are nononcogenic retroviruses which cause chronic diseases with long incubation periods. Visna virus, the prototype of this group, causes chronic pneumonitis and a progressive demyelinating disease in sheep months to years after the initial infection (24, 38, 39). Caprine arthritis-encephalitis virus (CAEV) is a related virus which causes leukoencephalitis in young goats and chronic arthritis in adult animals (5, 6). Long studied in animal models for demyelinating disease, visna virus and CAEV have assumed new importance because of the many similarities between these viruses and human T-cell lymphotropic virus type III (HTLV-III), the etiologic agent of acquired immune deficiency syndrome (AIDS) (2, 8, 26, 36). These viruses are unusual among retroviruses in their ability to replicate to high titers in tissue culture, ultimately killing their host cell. In contrast, replication of the lentiviruses is highly restricted in the infected host. Visna virus or CAEV antigen is seldom demonstrable in monocytes or macrophages, the target cell for the lentiviruses in vivo, even in chronically infected animals with debilitating disease (23, 24). Similarly, replication of HTLV-III appears to be highly restricted in the lymphocytes of AIDS patients (15).

Cloning of the HTLV-III, CAEV, and visna virus genomes has made it possible to compare the nucleotide sequences of these viruses and established that they are clearly related and distinct from the A, B, C, and D type retroviruses, as well as from the HTLV-I, HTLV-II, and bovine leukemia virus subfamily of retroviruses (4, 10, 11, 28, 42). One of the distinguishing features of the lentivirus-HTLV-III group appears to be the presence of two additional open reading frames between the *pol* and *env* genes. In HTLV-III one of these regions, termed the *tat*-III gene, has been shown to specifically increase the expression of sequences transcribed by the viral long terminal repeat (LTR) via a process termed *trans*-activation (1, 37, 40). The visna

virus genome contains similar open reading frames between the *pol* and *env* genes (42), and recently we have shown that gene expression directed by the visna LTR is also activated in *trans* in infected cells, although the magnitude of the *trans*-activation effect was considerably lower than was observed for HTLV-III (17, 41).

In this report we studied the regulation of expression of visna virus in more detail and extended our studies to CAEV to determine whether CAEV, like visna virus and HTLV-III, also exhibits the *trans*-activation effect. We believe this point is important because it may clarify whether *trans*-activation is a common feature of the lentiviruses and is necessary to cause disease. In addition, the presence or absence of the *trans*-activation effect might explain, in part, why some lentiviruses, such as visna virus, are highly cytolytic in tissue culture, while others, such as CAEV, are not. The generally higher replication rate of visna virus than of CAEV in vitro suggested to us that the CAEV promoter was either weaker than the visna promoter or that CAEV infection did not *trans*-activate expression of the CAEV genome.

We began our study by sequencing the CAEV LTR and determining the location of the start site for RNA transcription and the polyadenylation site. Then, the complete CAEV LTR was inserted into a plasmid so that it directed expression of the bacterial gene for chloramphenicol acetyltransferase (CAT). Our previous experiments on the regulation of visna virus expression were done with plasmids containing only the U3 region and a portion of the R region directing transcription of the CAT gene. Since transcribed sequences in the R region are now known to be important for *trans*-activation in HTLV-III (33), we constructed new plasmids containing the entire visna virus LTR directing expression of the CAT gene. These plasmids were transfected into a variety of cell types, either uninfected or infected with either visna virus or CAEV, and the activity of the promoters was

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FIG. 1. Nucleotide sequence of the CAEV LTR. The CAEV LTR was sequenced as described in Materials and Methods. The TATA box and polyadenylation signal are underlined. The positions of the 71-bp direct repeats are indicated by arrows. PPT, Polypurine tract; IR, inverted repeat; PBS, primer-binding site.

measured by subsequently assaying the CAT activity in cell extracts.

The results of the experiments described here suggest that CAEV infection does not *trans*-activate expression of its own genome, either in primary macrophages, which are the target cell for the virus in the infected animal, or in a variety of fibroblastic cells. In contrast, infection of the same cells with visna virus, a more rapidly replicating and cytopathic lentivirus, caused dramatic increases in gene expression directed by either the visna virus or CAEV LTR, with the magnitude of the *trans*-activation effect dependent on the cell type. Thus, the *trans*-activation effect does not appear to be a common property of the pathogenic lentiviruses.

MATERIALS AND METHODS

Sequencing. Cloning of the entire CAEV genome from unintegrated DNA has been reported elsewhere (28). The CAEV LTR was sequenced by subcloning the 0.6-kilobase (kb) *HindIII*–*HindIII* restriction fragment spanning the U3 region of CAEV and the 0.5-kb *HindIII*–*XbaI* fragment spanning the R and U5 regions into bacteriophage M13mp10 and M13mp11. Dideoxy sequencing was done as described by Sanger et al. (35), with the modification of Biggin et al. (3). The sequence of the CAEV LTR is shown in Fig. 1. Alignment of the sequences of the CAEV and visna virus LTRs, shown in Fig. 3, was done with the NUCALN program of Wilbur and Lipman (47).

Primer extension of CAEV RNA. Primer extension analysis was done on oligo(dT)-selected CAEV virion RNA to determine the location of the cap site. A 20-base-pair (bp) oligonucleotide primer complementary to the sequence GCGGCCGGTGTATCGGGAGT located in the CAEV U5

FIG. 2. Primer extension mapping of the 5' end of CAEV RNA. The location of the CAEV cap site was determined by primer extension as described in Materials and Methods. Primer extension of the CAEV RNA gave products of 123 and 124 bp as well as several minor shorter species. ³⁵S-dideoxy sequencing reactions on a CAEV *HindIII*–*AccI* fragment cloned into M13mp19, which spans the R-U5 region of CAEV, were run in parallel. From left to right, the lanes contain T, G, C, and A, followed by the primer extension product. Comparison of the primer extension and dideoxy sequencing products shows that the cap site is the first G nucleotide in the sequence CTTCAGAGTTC, 26 bp downstream from the TATA box.

region was chemically synthesized and purified by high-pressure liquid chromatography. This primer (30 ng) was combined with 1 µg of viral RNA in a total volume of 4 µl. This template-primer mix was heated to 100°C for 3 min, 1 µl of RNasin (25 U/µl; Promega-Biotech) was added, and this mixture was incubated for 30 min at room temperature. Following this incubation, 2 µl of [³⁵S]dATP (10 µCi/µl; New England Nuclear Corp.) and 2 µl of deionized distilled H₂O were added. A 4-µl amount of this solution was combined with 6 µl of primer extension buffer (10 mM Tris chloride, pH 8.3, 10 mM MgCl₂, 45 mM NaCl, 7.5 mM dithiothreitol, 9 µM dATP, and 375 mM each dCTP, dGTP, and dTTP), and this solution was incubated for 20 min at 42°C. Then, 4 µl of chase solution (1 mM solution of dATP, dCTP, dGTP, and dTTP in deionized distilled H₂O) was added. After an additional 20 min at 42°C the reaction was stopped and a portion was run on a 6.0% urea-acrylamide sequencing gel (Fig. 2).

Plasmid constructions. Plasmids expressing the CAT gene were constructed as shown in Fig. 4. The cloning of CAEV

DNA used for the construction of plasmids containing CAEV sequences has been reported elsewhere (28). Visna virus DNA used to construct pVIS-RU5-CAT was subcloned from a lambda clone of visna virus kindly provided by A. Haase (16). Other visna virus sequences were from DNA cloned in our laboratory (21). Plasmids were constructed by published procedures (19) and purified on CsCl gradients prior to use in transfections.

Plasmids containing the entire CAEV and visna virus LTRs 5' to the CAT gene were constructed (see Fig. 4) by inserting CAEV or visna virus sequences into pUC-CAT, a plasmid constructed in our laboratory, which contains the CAT gene placed immediately downstream from a pUC18 polylinker (hatched box) (31, 48). To construct pCAEV-LTR-CAT, an M13mp11 subclone of the *Hind*III-*Xba*I fragment spanning the R-U5 region of CAEV was digested with *Nar*I (which cuts at the 3' end of the CAEV LTR) and *Bgl*II (which cuts within M13 sequences). This fragment was gel purified and then ligated between the *Bam*HI and *Acc*I sites in the pUC-CAT polylinker to produce the plasmid pCAEV-RU5-CAT. (*Bam*HI ends are cohesive with *Bgl*II ends, and *Nar*I ends are cohesive with *Acc*I ends; prior to insertion of CAEV sequences, the *Hind*III site in pUC-CAT was destroyed by digestion with *Hind*III followed by treatment with *Escherichia coli* DNA polymerase Klenow fragment and religation). pCAEV-RU5-CAT was digested with *Hind*III, and an approximately 400-bp *Hind*III-*Hind*III restriction fragment spanning the U3 region of CAEV was ligated into this site to generate the plasmid pCAEV-LTR-CAT, which contains a complete CAEV LTR driving transcription of the CAT gene. The plasmid pVIS-LTR-CAT was constructed by first digesting a pUC18 subclone of the *Sst*I-*Sst*I fragment which spans the R-U5 region of visna virus with *Hind*III (which cuts within the visna virus insert approximately 100 bp downstream from the 3' end of the LTR) and *Bam*HI (which cuts within the pUC18 polylinker), purifying the approximately 250-bp piece generated, and ligating this piece between the *Hind*III and *Bam*HI sites of pUC-CAT to produce the plasmid pVIS-RU5-CAT. An M13mp10 subclone of the *Hind*III-*Sst*I fragment spanning the U3 region of visna virus was digested with *Hind*III, treated with *E. coli* DNA polymerase Klenow fragment, and ligated to *Bam*HI linkers. Following digestion with *Bam*HI and *Sst*I, the resulting 480-bp fragment was gel-purified and ligated between the *Bam*HI and *Sst*I sites of pVIS-RU5-CAT to produce the plasmid pVIS-LTR-CAT, which contains a complete visna virus LTR driving transcription of the CAT gene. The plasmid pCAEV-U3-CAT (not shown) was constructed by cleaving plasmid pSV2CAT, described elsewhere (13), with *Acc*I, treating with *E. coli* DNA polymerase Klenow fragment, and ligating on *Hind*III linkers. Following digestion with *Hind*III (which cleaves away all simian virus 40 promoter sequences) and purification of the vector, the approximately 400-bp *Hind*III-*Hind*III fragment which spans the U3 region of CAEV was ligated between the *Hind*III sites to produce plasmid pCAEV-U3-CAT. These constructions are illustrated in Fig. 4.

Transfections and CAT assays. Primary macrophages were prepared by purifying mononuclear cells from the blood of an uninfected goat by Ficoll-Hypaque centrifugation and then plating these cells on 35-mm plastic dishes. Nonadherent cells were washed away, and the adherent macrophages were grown in Dulbecco modified Eagle medium containing 20% lamb serum. Nearly confluent monolayers of sheep choroid plexus (SCP) cells (23), sheep alveolar macrophages (SAM) (9), primary goat macrophages, and goat synovial

membrane (GSM) cells (24) grown in 35-mm dishes were transfected by the DEAE-dextran technique coupled with a dimethyl sulfoxide shock (18, 20). Cells were infected with visna virus by adding visna virus strain 1514 at 24 h before transfection at multiplicities of 0.5 times the 50% tissue culture infective dose (TCID₅₀) (SCP and GSM cells) or 1.0 TCID₅₀ (macrophages). Macrophages and GSM cells were infected with CAEV by adding CAEV 24 h prior to infection at a multiplicity of 1.0 TCID₅₀. Cells to be transfected were washed with serum-free medium, and then DNA was added at a concentration of 3 µg/ml in 1 ml of serum-free medium containing 200 µg of DEAE-dextran per ml (at this DNA concentration, CAT activity is proportional to the amount of input DNA). After 1 h at 37°C the cells were treated with 1 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline (18) containing 10% dimethyl sulfoxide for 2 min at room temperature. The cells were washed once with phosphate-buffered saline, and then the original medium on the cells was replaced (modified Eagle medium containing 0.5% fetal bovine serum was used for GSM and SCP cells, Dulbecco modified Eagle medium containing 2% lamb serum was used for the macrophages). The cells were then incubated for 48 h at 37°C.

Cells grown in monolayers were harvested for the CAT assay by scraping. After being washed once with cold phosphate-buffered saline (pH 7.4), the cells were lysed by freeze-thawing three times in 60 µl of 250 mM Tris chloride (pH 7.8). Cell debris was pelleted by centrifugation, and 30 µl of the cellular supernatant was assayed for CAT activity as previously described by Gorman et al. (13). The percent acetylation of [¹⁴C]chloramphenicol (New England Nuclear) after various times was measured by separating the acetylated and unacetylated forms by thin-layer chromatography, followed by liquid scintillation counting of spots cut from the plate. The results are expressed as the percent conversion of [¹⁴C]chloramphenicol to its acetylated derivatives per hour by one half of a cell extract made from the cells growing in a 35-mm dish (Table 1).

RESULTS

Sequence analysis. The complete sequence of the CAEV LTR is shown in Fig. 1. Retroviral LTRs are bounded by short inverted repeats which contain the highly conserved dinucleotides TG at the 5' end and CA at the 3' end. In CAEV, 4-bp imperfect repeats (ACTG/CAGC) containing these highly conserved dinucleotides were found at the ends of the LTR. Retroviral genomes contain a short sequence immediately downstream from the 5' LTR, which serves as a primer-binding site for the synthesis of minus-strand viral DNA. In CAEV this 17-bp sequence is TGGCGCC CAACGTGGGG, which is complementary to mammalian tRNA_{1,2}^{Lys} (29). Visna virus contains this same sequence (42). The primer-binding sites of HTLV-III (30, 34, 45), equine infectious anemia virus (43), and mouse mammary tumor virus (MMTV) (7, 25) differ from the CAEV primer-binding sequence at five positions and are complementary to tRNA₃^{Lys}. The primer-binding site of a recently sequenced type D simian retrovirus is complementary to tRNA_{1,2}^{Lys} (27).

Purine-rich sequences located upstream from the 3' LTR of retroviruses serve as the initiation site for synthesis of plus-strand DNA. In CAEV a likely initiation site is the polypurine tract AAAAAAGAAAGGGTGG, which is identical to a sequence 5' to the visna virus LTR and similar to the polypurine tracts in HTLV-III (30, 34, 45) and MMTV

TABLE 1. Transcriptional activity of the CAEV and visna virus LTRs in infected and uninfected cells^a

Cells and infecting virus	CAT activity (% conversion/h)			
	pVIS-LTR-CAT	pCAEV-LTR-CAT	pCAEV-CAT	pRSV-CAT
SCP				
None	0.53	0.33	0.21	0.12
Visna virus	6.64	1.85	1.13	0.12
GSM				
None	0.08	0.06	0.04	0.04
CAEV	0.26	0.05	0.05	0.04
Visna virus	6.84	0.93	0.66	0.04
SAM				
None	27.17	8.97	5.98	0.85
CAEV	23.78	10.36	4.79	1.13
Visna virus	42.63	13.10	20.06	1.16
Primary goat macrophages				
None	3.46	0.52	0.78	0.25
CAEV	6.50	1.62	2.90	0.40
Visna virus	22.74	4.02	21.20	0.26

^a Cells were transfected with the indicated plasmids and were then assayed for CAT activity 48 h later as described in Materials and Methods. The results are expressed as the percent conversion of [¹⁴C]chloramphenicol to acetylated derivatives per hour by one-half of an extract from the amount of cells growing in a 35-mm dish. Extracts of a given cell type contained equal amounts of protein, whether infected or uninfected. The incubation times used (3 h for GSM cells, 1 h for the other types) gave CAT activity measurements which were within the linear range of the assay. The values reported are the averages of three separate transfections. In most cases, variation between values was less than 30%. The construction of pRSV-CAT has been reported previously (12).

(7). From the positions of the 4-bp imperfect repeats discussed above, and the positions of the putative plus and minus strand initiation sites, we can define the boundaries of the CAEV LTR and deduce its overall length to be 450 bp. This is similar to the size of the visna virus LTR (414 bp) (42) but is considerably smaller than the LTRs of other sequenced mammalian retroviruses, including HTLV-III (634 to 638 bp) (30, 34, 45) and MMTV (1,327 bp) (7).

The start site of RNA transcription was mapped by primer extension to the G nucleotide 26 bp downstream from the TATA sequence (Fig. 2). These results are consistent with strong-stop experiments which shown that the R-U5 region of CAEV is approximately 153 bp long (J.E.C., unpublished data). The location of the R-U5 boundary of the CAEV LTR was determined by dideoxy sequencing of oligo(dT)-selected viral RNA with avian myeloblastosis virus reverse transcriptase. Poly(dT) primers ending with C, G, or A (New England Biolabs) were used to prime the reverse transcriptase. The primer poly(T-A) gave no discrete bands on a sequencing gel, the poly(T-G) primer gave a single sequence which did not correspond to LTR sequences (presumably this primer initiated synthesis elsewhere on the CAEV RNA), and the poly(T-C) primer gave multiple superimposed sequences (data not shown). Thus, it appears that the R region of the CAEV LTR ends in the sequence GA, with the A nucleotide located at position +85, 21 bp downstream from the polyadenylation sequence AATAAA. From the positions of the cap site and polyadenylation site in CAEV, we conclude that the CAEV U3 region is 78 bp long, the R region is 85 bp long, and the U5 region is 78 bp long. These dimensions resemble those of the visna virus LTR (U3 = 255, R = 97, and U5 = 62 nucleotides) (42).

Sequences which govern the initiation and rate of RNA synthesis are contained within the U3 region of the retroviral LTR (44). The CAEV U3 region contains elements which are commonly found in viral and eucaryotic promoters. A Goldberg-Hogness box (TATAA) is found in the CAEV promoter 26 bp upstream from the RNA initiation site. In addition, the CAEV U3 region contains a pair of 71-bp direct repeats located 108 bp upstream from the cap site, as well as other shorter repeated sequences both within and outside these direct repeats. Although the CAEV U3 region lacks the "core" enhancer sequence (GTGGTTTG or GTGGAAAG) found in many viral and eucaryotic enhancer sequences (46), it is likely, because of their location, that at least part of these repeated sequences act as an enhancer element.

Homology with other retroviruses. A computer-assisted search of other retroviral LTR sequences revealed that the CAEV LTR only had significant homology with the visna virus LTR, with approximately 50% overall homology. Certain regions within the CAEV and visna LTRs were more highly conserved. The aligned LTR sequences of CAEV and visna virus are shown in Fig. 3. The 71-bp direct repeats of the CAEV U3 region contains a shorter sequence at their 3' ends which is closely related to the 43-bp repeats in the visna virus U3 region. Farther downstream from the direct repeats was a 13-bp sequence, located approximately 60 bp from the cap site, that is identical to sequences in the same location in the visna virus LTR. The visna virus and CAEV LTRs contains the sequence TATATAA 16 and 26 bp, respectively, upstream from the cap site. Sequences flanking these conserved promoter elements were markedly divergent. The R region of CAEV also showed short regions of homology with visna virus, most strikingly in the identical 10-bp sequence AATAAAGGAGT located 22 bp upstream from the polyadenylation site in CAEV and 14 bp upstream from the polyadenylation site in visna virus. The U5 region of CAEV was highly related to the visna U5 region, particularly at the 3' end (57% overall homology).

The transcriptional activity of the CAEV U3 region and entire LTR was tested by fusing these regions with the CAT gene and transfecting these plasmids into a variety of cell types (Fig. 4). Assays for CAT activity directed by these plasmids were done 48 h after transfection, and the percent conversion of [¹⁴C]chloramphenicol to its acetylated derivatives per hour was calculated. For comparison, plasmids containing the CAT gene driven by either the visna virus or RSV LTR (12) were transfected into cell cultures grown in parallel. These results for infected and uninfected cell types are shown in Table 1. In all cell types tested the CAT activity directed by the CAEV LTR was greater than that directed by the RSV promoter but substantially less than that directed by the visna virus LTR. The transcriptional activity of the CAEV U3 region alone was comparable to that of the entire CAEV LTR in all uninfected cell types tested. In uninfected SCP and GSM cells the CAEV LTR was only slightly less active than the visna virus LTR (0.33 versus 0.53% conversion in the SCP, 0.06 versus 0.08% conversion per h in the GSM cells). Differences in activity between the CAEV and visna virus promoters were more marked in the sheep macrophages. The CAEV LTR was threefold less active than the visna virus LTR in SAM cells and roughly sixfold less active than the visna LTR in primary goat macrophage cultures.

The relative activities of the CAEV, visna virus, and RSV promoters was not significantly affected by infection by CAEV, with the exception of the primary macrophages. CAEV infection of these cells resulted in modest (two- to

FIG. 3. Alignment of the CAEV and visna virus LTRs. Sequences of the CAEV LTR and visna virus LTR determined in our laboratory were aligned by using the NUCALN program of Wilbur and Lipman (47). Homologous nucleotides between CAEV (top line, C), and visna virus (bottom line, V) are indicated with dots. The boundaries of the U3, R, and U5 regions are indicated.

FIG. 4. Construction of plasmids expressing the CAT gene. Details of the construction of these plasmids are given in Materials and Methods.

threefold) increases in the activity of the visna virus and CAEV promoters. CAEV infection of GSM cells had no effect on the activity of the CAEV LTR even when the cells were transfected at later times after CAEV infection (3 to 5 days), when the majority of cells have fused into multinucleated giant cells as a result of CAEV replication (data not shown). In contrast, infection of GSM cells, SCP cells, or primary macrophages with visna virus resulted in dramatic increases in the CAT activity directed by either the CAEV or visna virus promoter. The most dramatic increase in CAT activity was observed in visna virus-infected GSM cells, cells in which the basal level of activity of the CAEV and visna virus LTRs is low. Infection of these cells with visna virus resulted in a 16-fold increase in CAT activity directed by the CAEV LTR and an 86-fold increase in activity directed by the visna virus LTR (Fig. 5).

In SCP cells infection with visna virus increased the activity of the CAEV LTR or U3 region by roughly 6-fold, while the visna virus LTR activity was increased by 13-fold. Visna virus infection of primary macrophages increased the activity of the CAEV and visna virus LTRs by roughly seven- to eightfold. Curiously, the CAEV U3 region was more dramatically affected by visna virus infection than was the entire LTR in both SAM and primary macrophage

cultures. In primary macrophages, for example, the activity of the CAEV LTR was increased 8-fold by visna virus infection, while the activity of the U3 region alone was increased by 26-fold to roughly the same activity as the visna virus LTR (approximately 21% acetylation). This activation of gene expression by visna virus infection appears to be specific for the visna virus and CAEV promoters, as the transcriptional activity of the RSV promoter was not affected by visna virus infection in any of the cell types tested. DNA slot-blots were done on both fibroblastic cells and sheep macrophages to confirm that the increases in CAT activity in visna virus-infected cells were not the result of increased plasmid DNA uptake or stabilization in infected cells (J.L.H., unpublished data).

DISCUSSION

Nucleotide sequencing of the CAEV LTR has revealed that CAEV and visna virus have considerable sequence homology in specific portions of their LTRs, notably in the 3' portions of the 71-bp repeats of the U3 region, in short sequences further downstream in the U3 region, in the TATA and polyadenylation sequences, and throughout

FIG. 5. CAT assay of uninfected and CAEV- or visna virus-infected GSM cells. Monolayers of GSM cells were either mock infected or infected with CAEV or visna virus. After 24 h the cells were transfected with pVIS-LTR-CAT (lanes 1), pCAEV-LTR-CAT (lanes 2), pCAEV-U3-CAT (lanes 3), or pRSV-CAT (lanes 4), and after incubation at 37°C for 48 h, CAT activity in cell extracts was assayed as described in Materials and Methods.

much of the U5 region. This finding supports the close genetic relationship between CAEV and visna virus that has been suggested on the basis of heteroduplex analysis (11) and limited sequence analysis of other parts of the viral genome (4). Except for short stretches of local homology, such as sequences containing the TATA box, CAEV is not significantly homologous to HTLV-III in the LTR region. However, as previously noted, CAEV and HTLV-III (as well as visna virus and MMTV) have similar polypurine tracts adjacent to the 3' LTR, and both viruses have tRNA^{Lys} primer-binding sites, a finding which suggests at least a distant evolutionary relationship between HTLV-III, the lentiviruses, and MMTV.

Our previous work on visna virus showed that visna virus infection of cells *trans*-activated gene expression directed by the visna virus U3 region (17). The magnitude of this *trans*-activation of the U3 region was small compared with that observed for the entire HTLV-III LTR in HTLV-III-infected cells (41). Subsequently we constructed plasmids containing the entire visna virus LTR driving transcription of the CAT gene and found that in certain cells, such as GSM cells, visna virus infection increased the activity of the visna virus LTR much more dramatically. Surprisingly, in the experiments described here, we found no evidence for analogous *trans*-activation of the CAEV LTR in CAEV-infected cells. The DNA used to construct the plasmids pCAEV-CAT and pCAEV-LTR-CAT was derived from an infectious clone of CAEV (28), so it is unlikely that the CAEV LTR is defective. While we cannot exclude the possibility that *trans*-activation of CAEV expression occurs in cell types other than the ones studied here, we think it is most likely that the CAEV genome either lacks or contains a defective *trans*-activator gene.

The lack of *trans*-activation of the CAEV LTR in CAEV-infected cells may account for the slower replication rate of CAEV than of visna virus in fibroblastic cells. The CAT activity directed by the visna virus LTR in visna virus-infected GSM cells was more than 100-fold higher than the activity of the CAEV LTR in CAEV-infected cells. These

measurements are consistent with the approximately 100-fold-higher titers of visna virus measured in GSM supernatants (22). Visna virus and CAEV replicate to comparable titers in sheep and goat macrophages (O. Narayan, unpublished data), and in accordance with this observation, the CAEV and visna virus LTRs had more similar activities in macrophages infected with either CAEV or visna virus. In these cells, both promoters had higher basal levels of activity and were less dependent on viral infection for maximal activity than in GSM cells. The relatively small difference in the activity of the CAEV and visna virus LTRs in SCP cells, however, does not explain why visna virus replicates in these cells to 1,000-fold-higher titers than CAEV (O. Narayan, unpublished data). This suggests that events earlier in the replicative cycle of the virus, such as viral uncoating, reverse transcription, or proviral DNA integration, may be blocked in SCP cells.

The molecular mechanism of *trans*-activation of viral gene expression in visna virus-infected cells is not known. Preliminary experiments in our laboratory indicate that visna virus infection of GSM cells increases the steady-state levels of genes transcribed by the visna virus LTR, but not enough to account for the increases in CAT activity that were observed. This suggests that enhanced translation of viral transcripts may be involved in the *trans*-activation effect, as has been shown for HTLV-III (32). If this is the mechanism for *trans*-activation, it is not clear why visna virus infection affects the activity of the visna virus and CAEV U3 regions, whose transcripts contain few if any viral sequences. Further work is needed to determine if the mechanism of *trans*-activation by visna virus is the same as that of *trans*-activation by HTLV-III.

Our experiments suggest that *trans*-activation may not be a general property of the lentiviruses. As CAEV is capable of causing debilitating and life-threatening infections and apparently does not *trans*-activate, this suggests that *trans*-activation may not be an important determinant of lentivirus pathogenicity in the infected host. Studies of equine infectious anemia virus and other lentivirus isolates should

help clarify the significance of the *trans*-activation effect in vivo.

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