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Biological and Genetic Differences Between Lung- and Brain-Derived Isolates of Maedi-Visna Virus

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Abstract. During the epidemic caused by maedi-visna virus (MVV) of sheep in Iceland, the pulmonary affection, maedi, was the predominant clinical manifestation. In some flocks, however, a central nervous system (CNS) affection, visna, was the main cause of morbidity and mortality. As there is only one breed of sheep in the country, host factors did apparently not play an important role in the different clinical manifestations. To obtain some information on possible viral genetic determinants of neurotropism and neurovirulence we studied both phenotypic and genotypic properties of two maedi-visna virus strains; a strain that was originally isolated from the brain of sheep with encephalitis (visna), and another strain isolated from the lungs of a sheep suffering from pneumonia (maedi). The brain isolate was found to grow faster in sheep choroid plexus cells than the lung isolate, whereas the growth rate in macrophages was similar for the maedi and visna virus strains. Intracerebral inoculation indicated that the visna virus isolate induced more severe brain lesions than the maedi isolate. In addition, a pathogenic molecular clone derived from a visna strain (KV1772kv72/67) was tested for growth in sheep choroid plexus cells and macrophages. The molecularly cloned virus retained the fast growth rate in choroid plexus cells. The nucleotide sequence of the *env* gene and the U3 of the LTR was determined for the maedi strain and compared to that of the visna strains. There was an 11.7% difference in deduced amino acid sequence in the Env protein and a 6% difference in the LTR. The molecular clone KV1772kv72/67 will be a useful reagent for characterization of viral determinants of cell tropism *in vitro* and possibly neurovirulence *in vivo*.

Key words: lentivirus, maedi-visna virus, neurovirulence, cell tropism

Introduction

Maedi-visna virus (MVV), one of the lentivirus subgroup of retroviruses (1) has a broad range of organ tropism, i.e. it causes encephalitis (visna), pneumonia (maedi), mastitis and arthritis in sheep (reviewed in 2). Maedi-visna virus was introduced to Icelandic sheep with the importation of apparently healthy sheep of the Karakul breed in 1933. During the epidemic in Iceland the main clinical manifestation was maedi. However, in some sheep flocks in a restricted area of the country, visna was the main cause of morbidity and mortality (3). The occurrence of severe CNS disease caused by MVV in a restricted area in Iceland has been a puzzle. Host factors apparently do not play a major role as there is only one breed of sheep in Iceland that has lived in almost total isolation for 1100 years (3). Epidemiological and experimental data indicate that differences in organ tropism may be due to viral genetic determinants. Results of earlier *in vitro* experiments support this

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GenBank accession numbers: The *env* sequence of MVV strain KM1071: U51910 U3 of the LTR in MVV strain KM1071: U52358

view. Thus virus isolates from visna-affected brains were found to grow nearly twice as fast as isolates from maedi-affected lungs in choroid plexus cells (4).

The primary target cells of MVV *in vivo* are considered to be of the monocyte lineage (5–7) although viral RNA and DNA (8) as well as viral proteins (9) have been detected in other cell types including neuroglia, endothelial cells and lymphocytes. In infections with related lentiviruses, such as HIV-1 and SIV, brain derived virus isolates have been shown to be macrophage-tropic by a number of laboratories (10–12). Viral genetic determinants in the *env* gene seem to play an important role in the cellular tropism of the virus (13–18) and the long terminal repeats have also been shown to vary in the efficiency of directing replication in various cell types (19,20).

In this study we examined a number of biological properties of two closely related brain isolates, one of which is molecularly cloned, and a lung isolate of MVV in order to find out whether the brain isolates retain a neurovirulent property on transmission *in vivo*, and whether there is a difference in cell tropism *in vitro*. The *env* gene and the U3 of the LTR of the maedi strain KM1071 were sequenced and compared with the published sequence of the visna strain KV1772 (21) to obtain information on possible viral genetic determinants of neurovirulence and cell tropism.

Materials and Methods

Virus Strains and Cells

Maedi virus strain KM1071 was isolated in 1962 from the lungs of a sheep suffering from maedi, and has been through relatively few passages in tissue culture. Visna virus strain KV1514 is a descendant of a virus that was isolated from the brain of a sheep suffering from visna (22). This strain has been passaged extensively in sheep and tissue culture. Visna virus strain KV1772 is of the same lineage as KV1514 and they are 99% identical in nucleotide sequence (21). Strain KV1772kv72/67 is a pathogenic molecular clone derived from strain KV1772. Virus was propagated in monolayers of sheep choroid plexus cells (SCP) (1) and macrophages. Choroid plexus cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagles medium (DMEM) supplemented with 200 units/ml penicillin, 100 units/ml streptomycin, 2 mM glutamine and either 10% lamb serum (growth medium) or 1% lamb serum (maintenance medium).

Leukocytes were isolated from heparinized peripheral blood of healthy, uninfected sheep on a density gradient, Histopaque—1077 (Sigma Diagnostics). They were suspended in growth medium with 10% lamb serum and 5×10^{-5} M mercaptoethanol added, and counted and distributed at a density of 12×10^6/ml into 4×1 ml chamber with 18 mm^2 tissue culture slides (Permanox, Nunc, Inc). Cells were allowed to settle at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. Then the supernatant and unattached cells were removed and the slide washed twice energetically with PBS before adding 1 ml of new growth medium to each chamber. Adherent cells were further incubated for at least 7 days before they were infected.

Virus Titration

The viruses were assayed by endpoint titration in roller tube cultures or 96-well flatbottomed tissue culture plates. Final readings of cytopathic effects were made after 2–4 weeks. The infectivity titres were calculated by the Reed-Muench method (23). Alternatively virus growth was monitored by assaying the supernatants of infected cells for RT activity. Viral particles from 0.5 ml of cell-free supernatants from infected cells were pelleted at 70,000 rpm for 10 min in a Beckman TLA 100.4 rotor.

Reverse Transcriptase Assay

Reverse transcriptase (RT) was determined as described previously (24). Briefly, 0.5 ml of cell-free supernatants from infected cells were pelleted at 70,000 rpm and 4°C for 10 min in a Beckman TLA 100.4 rotor . The pelleted virus was resuspended in 10 µl of NTE (100 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA) containing 0.1% Triton X-100. This disrupted virus was incubated at 37°C for 1 h in a 50-µl reaction mixture containing 50 mM Tris-HCl (pH 7.8), 20 mM $MgCl_2$, 2 mM dithiothreitol, 20 mM KCl, 0.25 optical density unit each of poly (rA) and oligo (dT_{12-18}) per ml, and 2.5 µCi of [^{3}H]TTP (10–25 Ci/mmol; NEN). The reaction mixture (50 µl) was applied to DE81 filter paper discs (Whatman) and air dryed. The filters were washed three times in 5% Na_2HPO_4, once in H_2O, and at last in 70% ethanol. After drying, the filters were placed in vials with

Ultima Gold (Packard) and radioactivity was measured using a Packard Tri-Carb liquid scintillation counter.

Experimental Animals and Infection

Four outbred female Icelandic sheep, 7–12 months old, were infected with 0.3 ml of visna virus strain KV1514, titer $10^{6.5}$ $TCID_{50/}{}^{ml}$ and three with maedi strain KM1071, titer $10^{6.4}$ $TCID_{50/}{}^{ml}$. The inoculations were done intracerebrally as described previously (25). The sheep were sacrificed three months post infection. The intracerebral inoculations were done in Vetenarcol anaesthesia.

Virus Isolation

Blood was drawn at regular intervals during the experimental period and at sacrifice. Buffy coat cells were tested for virus by inoculation onto SCP monolayers. Spinal taps were done repeatedly during the experimental period and at sacrifice. Cells were counted and virus isolated from the spinal fluid by inoculations onto SCP monolayers. At sacrifice the following organs were tested for virus by explantation: CNS (3 samples from the brain, and 3 from different levels of the spinal cord), lungs (1 sample from each lobe), spleen, bone marrow, cervical, mediastinal and mesenterial lymph nodes.

Histology

The following organs were sampled and processed for histological examination: Brain (left half), spinal cord (cervical, midthoracic and midlumbar), lungs (1 block from each lobe), spleen, bone marrow, lymph nodes (cervical, mediastinal and mesenterial), thymus, heart, kidneys, liver, adrenal gland, mammary gland, and synovia. The samples were fixed in 10% phosphate buffered formalin, embedded in paraffin and sections cut at 3–5 microns. The brain was cut at 9 standard levels. The sections were routinely stained with haematoxylin-eosin but in addition lymphoid tissues were stained with Giemsa, and selected sections from the CNS with Klüver-Barrera stain for myelin.

The brain lesions were graded on a scale 0–6 as described previously (Pétursson et al., 1976) with a slight modification: 1, definite minimal lesions (2–3 of the following histological changes: periventricular inflammation, perivascular cuffs in white and/or gray matter, glial nodules, leptomeningitis, inflammation of the choroid plexus); 2, similar to 1 but more intense or widespread; 3, subconfluent but extensive periventricular lesions with some involvement of the white matter; 4, extensive confluent periventricular inflammation with some confluent involvement of the white matter; 5, as in 4 plus small necrotic lesions; 6, most severe cases of visna with more extensive necrosis. The lesions were assigned an intermediate grade, i.e. 0.5, if lesions were not observed in all main areas.

Cloning and Sequencing

Hirt DNA from strain KM1071 was isolated (26) from SCP cells after 3 days of infection. The DNA was digested with *Sac*I and cloned into lambda gt11. The library was screened with ^{32}P-labeled 8.6 kb *Sac*I fragment of visna virus. Of 48 positive clones, only 3 had the 3′ end of the genome intact. One of these was subcloned into pUC 18 and M13 for DNA sequencing. Sequencing was performed by the dideoxy-nucleotide chain termination method (27) using Sequenase 2.0 as specified by the manufacturer (United States Biochemicals). Every base was sequenced at least three times.

Amplification of Sequences in the env Gene and LTR

For PCR amplification in the LTR the following primer set was used: 5′-GATTATCATGGGAATTGTAGG-3′ and 5′-BioCTTTCCTTCGAGCTCTCCA for subsequent purification of single strands using strepavidin-coated magnetic beads (Dynal), or 5′-GATTATCATGGGAATTGTAGG-3′ and 5′-CCAGGCAAGCTCAGATATCA-3′ for subsequent cutting with *Sac*I and *Hind*III and cloning into M13 phage. Amplification of the region containing the premature termination in the *env* gene was carried out using the following primer set: 5′-GCAAAATACAGTTGTGAGAGTA-3′ and 5′-TGGTCTCGGTGTCGCAAA-3′. The PCR was performed by using *Taq* DNA polymerase and subjecting the samples to 30 cycles of denaturing at 94°C for 30 s, annealing at 53°C for 30 s and extension at 72°C for 1 min.

The region between the bases 7736 and 7979 was cut with *Xba*I and *Pst*I, cloned into M13 and sequenced.

The error rate of *Taq* DNA polymerase has been estimated at 2×10^{-4} nucleotides/cycle. This error rate is not likely to affect our results, since we are

looking for specific changes, i.e. a specific amino acid change in the *env* gene and a duplication in the LTR, and the PCR products were sequenced directly as well as cloned in M13.

Results

Growth Properties of Maedi Strain KM1071 and Visna Strains KV1514 and KV1772kv72/67 in SCP Cells and Sheep Macrophages

SCP cells were infected with the virus strains at various multiplicities of infection (m.o.i.), ranging from 0.5–10. Samples were taken at regular intervals for titration and RT assay. Fig. 1a shows that there is an initial lag in the growth of the maedi strain KM1071, it grows slower and to a slightly lower titer than the visna strains KV1514 and KV1772kv72/67. The average time for the visna strains to reach a titer of $10^{6.5}$/ml was 100 h when inoculated at m.o.i. 0.5 whereas the maedi strain reached the same titer in 180 h. At an m.o.i. of 10 the average time for the visna strains to reach a titre of $10^{6.5}$/ml was 28 h and 36 h for the maedi strain (data not shown). This difference between the visna strains on the one hand and the maedi strain on the other was consistent regardless of whether the inoculum was determined by RT units or end-point titration. We were not able to find any consistent difference in the growth rates of the three strains in macrophages (Fig. 1b,d).

Intracerebral Inoculation

Virus isolations. Four sheep were infected intracerebrally with visna virus strain KV1514 and three sheep were infected with maedi strain KM1071. At sacrifice three months post infection virus was recovered from several organs, including brain, lungs, spleen, lymph nodes and bone marrow. The total frequency of virus isolation was comparable in sheep infected with visna strain KV1514 and those infected with maedi strain KM1071 except that virus was recovered more

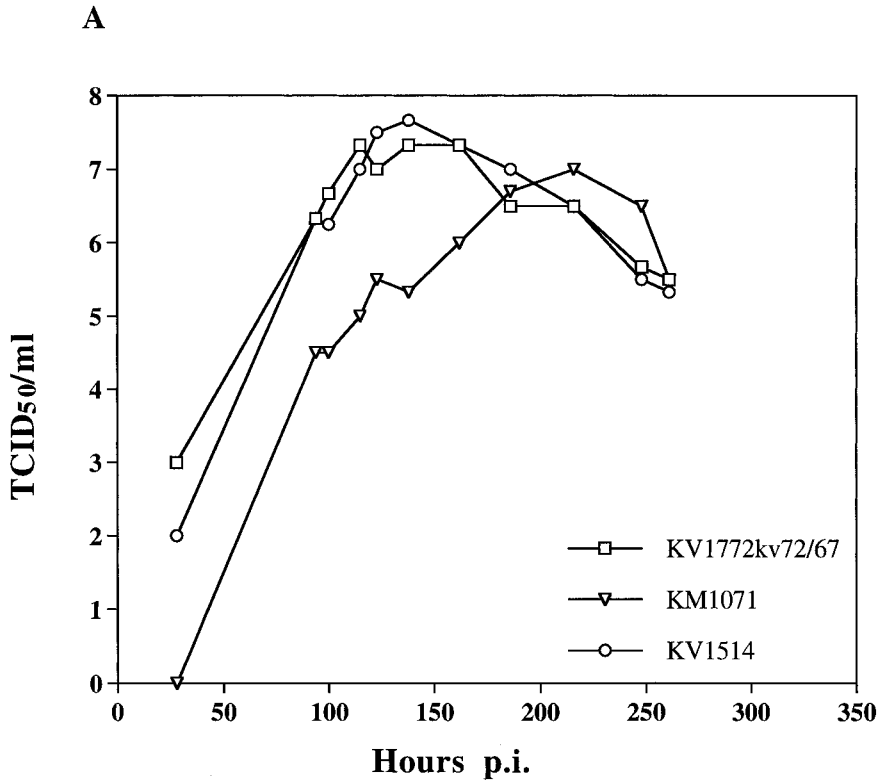
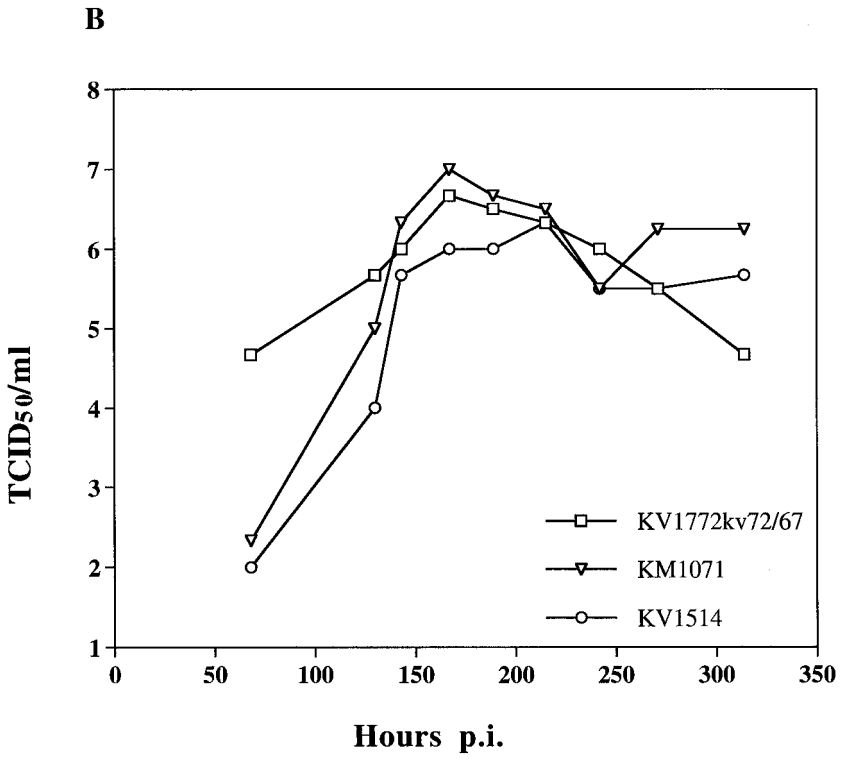
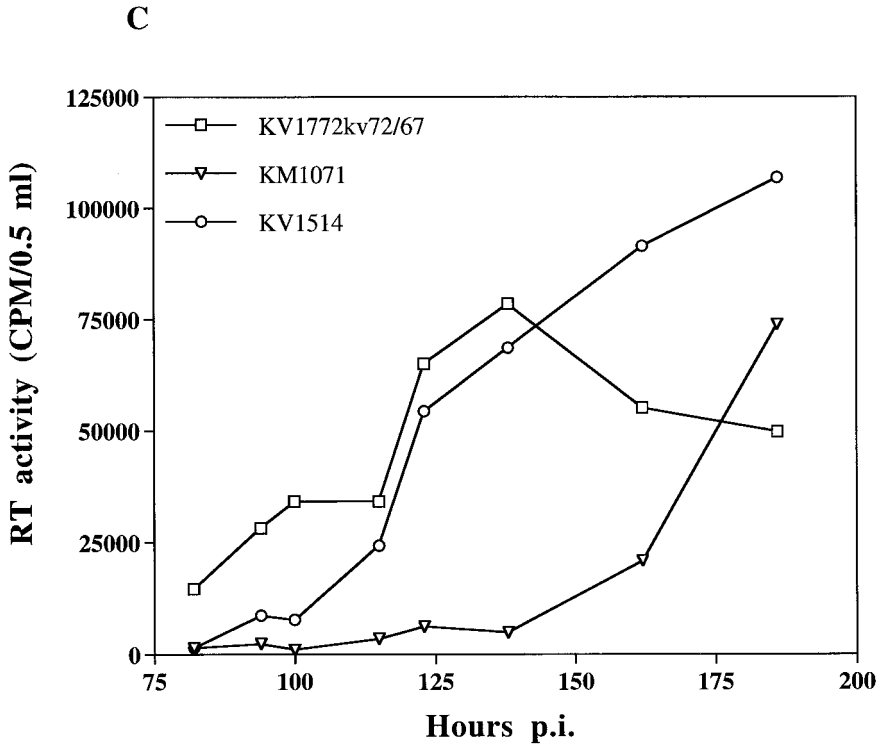


Fig. 1. Growth curves of visna and maedi strains in sheep choroid plexus cells (a,c) and in macrophages (b,d) measured by titration (a,b) and reverse transcriptase (c,d) . The cells were infected at an m.o.i. of 0.5.





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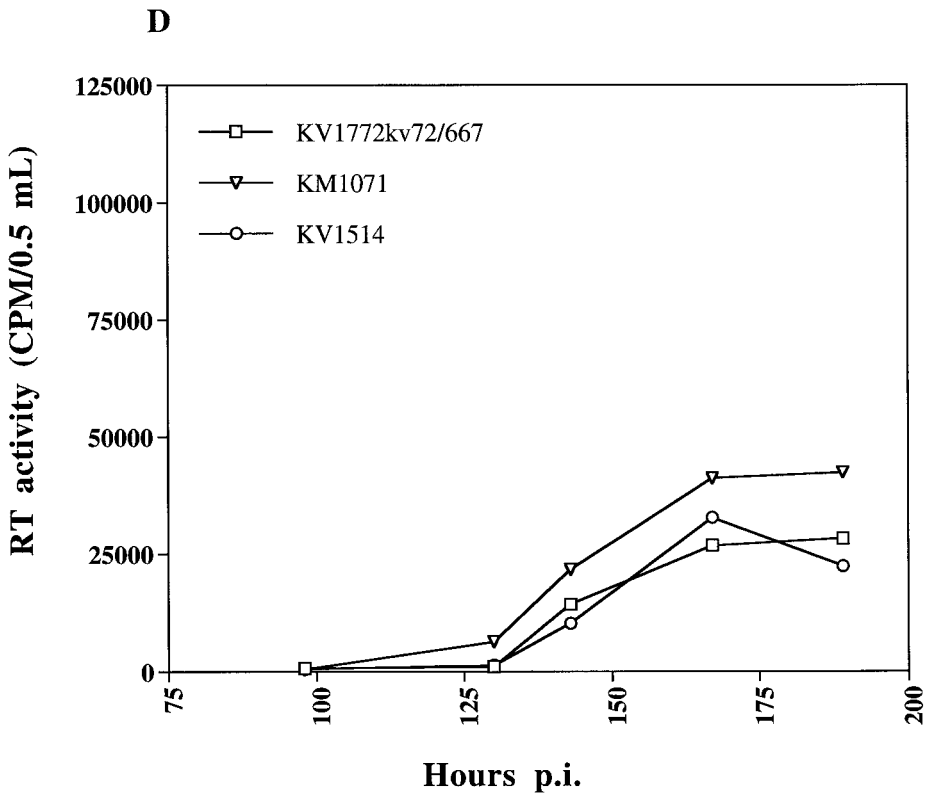


Fig. 1. (Continued)

frequently from the CNS in the visna-infected group (Table 1).

Histopathology. Definite visna lesions were found in the brain of all 4 sheep infected with visna virus KV1514. The lesion grades varied from 2 to 5, the average grade of lesions was 3.5 (Table 1). In those infected with the maedi strain KM1071 definite visna lesions were found in 1 out of 3. The average lesion grade was 0.7 (Table 1). Furthermore CSF cell counts were significantly higher in the visna infected group both one month p.i. and at sacrifice (Table 1). Fig. 2 shows the most severe brain lesions found in the sheep infected with maedi strain KM 1071 (a–d) and with visna virus strain KV1514 (e–h). The brain lesions showed the typical location and character of visna lesions (Fig.2). They consisted mainly of periventricular inflammation, sometimes extending into the adjacent white matter and leading in the most severe cases to confluent inflammation accompanied by myelin-breakdown. Leptomeningitis of a mild to severe degree was present and in the most severe cases occasional glial nodules and/or inflammatory foci were observed in the cortex (Fig. 2). Inflammatory infiltration of the choroid plexus was commonly observed, occasionally very prominent with formation of active lymphoid follicles.

Other organs did not show any specific changes that could be related to the infection with maedi or visna virus.

Nucleotide Sequence of the env Gene

The nucleotide sequence of the *env* gene in maedi strain KM1071 is shown in Fig. 3 and compared to the sequence of visna strain KV1772kv72/67 (Andrésson et al., 1993). This comparison revealed an overall difference of 8% in nucleotides and 11.7% in amino acids. There are 26 potential glycosylation sites in the Env protein of maedi KM1071, 21 in the surface glycoprotein and 5 in the transmembrane protein, whereas there are 29 potential glycosylation sites in visna KV1772kv72/67. Twenty-three of these sites are common to the two virus strains, and one is shifted by

Table 1. Comparison of intracerebral inoculation of visna virus KV1514 and maedi virus strain KM1071. Three months post infection

	Virus Isolations[1]						
Sheep No.	Blood	Lymph.	Lungs	CSF	CNS	CSF Cells	CNS Lesions Grade[2]
1532	0/2	2/2	—	0/2	2/3	434[3]-97[4]	3
1533	0/2	1/2	0/1	0/2	1/2	198-113	2
1534	0/2	2/2	0/2	0/2	2/2	— -109	4
1535	0/2	1/2	0/2	1/2	1/2	393-57	5
Total	0/8	6/8	0/5	1/8	6/9		
average	0%	75%	0%	12.5%	66.7%	341-94	3.5
1878	1/4	2/2	1/2	0/2	1/3	63-12	±
1879	0/4	2/2	0/2	0/2	1/3	11-22	±
1880	0/4	2/2	0/2	1/2	1/3	173-14	2
Total	1/12	6/6	1/6	1/6	3/9		
average	8.3%	100%	16.7%	16.7%	33.3%	82-16	0.7

Sheep no. 1532–1535 were infected with visna strain KV1514.
Sheep no. 1878–1880 were infected with maedi strain KM1071.
[1]Number positive/number tested. [2]Lesions graded on a scale 0–6. [3]One month post infection. [4]Three months post infection.

one amino acid (Fig. 3). Constant and variable regions were identified as described previously (28). A highly variable region at nt 7565–7717 near the carboxyl end of the extracellular glycoprotein carries two deletions of five and one amino acids. The regions coinciding with *rev* are also very variable (Fig. 4). A transition at nt position 7790 creating a premature termination codon was found in the clone of maedi KM1071 that was sequenced. Direct sequencing of a PCR product of this region in the Hirt supernatant revealed glutamine at this site as in visna KV1772.

Nucleotide Sequence of U3 of the Long Terminal Repeats (LTR)

U3 of the LTR of the maedi strain KM1071 was 6% divergent from visna KV1772. In the clone that was sequenced there was only one copy of a 43 bp repeat which is present in visna KV1772 and there was a 9 bp deletion in this region as well (Fig. 5). PCR products of the Hirt supernatant of maedi virus 1071 revealed that about half of the viral population had molecules of a size corresponding to two copies and the other half one copy of the 43 bp element.

Discussion

In this study we addressed the question whether strains of the ovine lentivirus MVV isolated from lungs (a maedi strain) and brain (a visna strain) are different with respect to neurovirulence and cell tropism and whether a molecular clone of a brain isolate can be used to map these phenotypic differences. Furthermore we have sequenced parts of the viral genome of the maedi strain and compared it to known sequences of the visna virus strain KV1772 (21).

Our findings *in vivo* indicate that there is a difference in neurovirulence between the maedi strain KM1071 and the visna strain KV1514. The visna virus strain KV1514 induced more severe CNS lesions than the maedi strain KM1071 and the CSF cell counts, another indicator of lesion activity in the brain, were higher in the visna virus infected group. There was, however, no qualitative difference in the lesion pattern between those infected with the maedi strain and those infected with the visna strain. The lesions were identical to those already described as characteristic for visna (2). Since the neurovirulence does not seem to be an all or none phenomenon, the number of sheep (4 for visna and 3 for maedi) are too low to draw statistically significant conclusions about

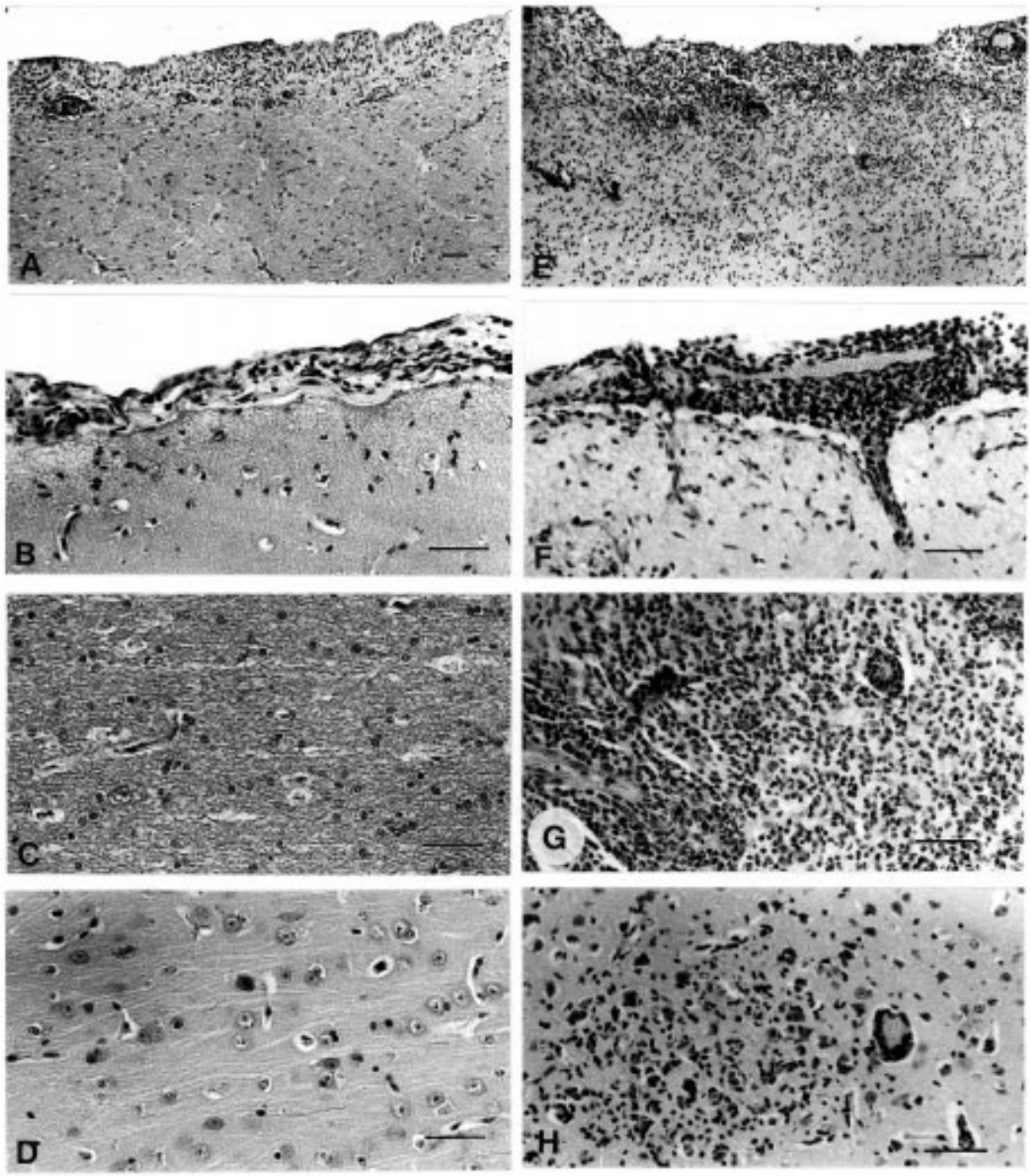


Fig. 2. Comparison of brain lesions in sheep infected by i.c. route with maedi strain KM1071 (a–d), lesion grade 2 and with visna virus strain KV1514 (e–h), lesion grade 5. a) Mild subconfluent periventricular inflammation; e) Severe confluent periventricular inflammation extending into adjacent white matter; b) Discrete infiltration of meninges; f) Severe meningitis; c) Normal white matter; g) Severe inflammation of white matter with myelin breakdown; d) Normal cortex; h) Perivascular infiltrates and glial nodule in cortex. H.E., bar 50 µm.

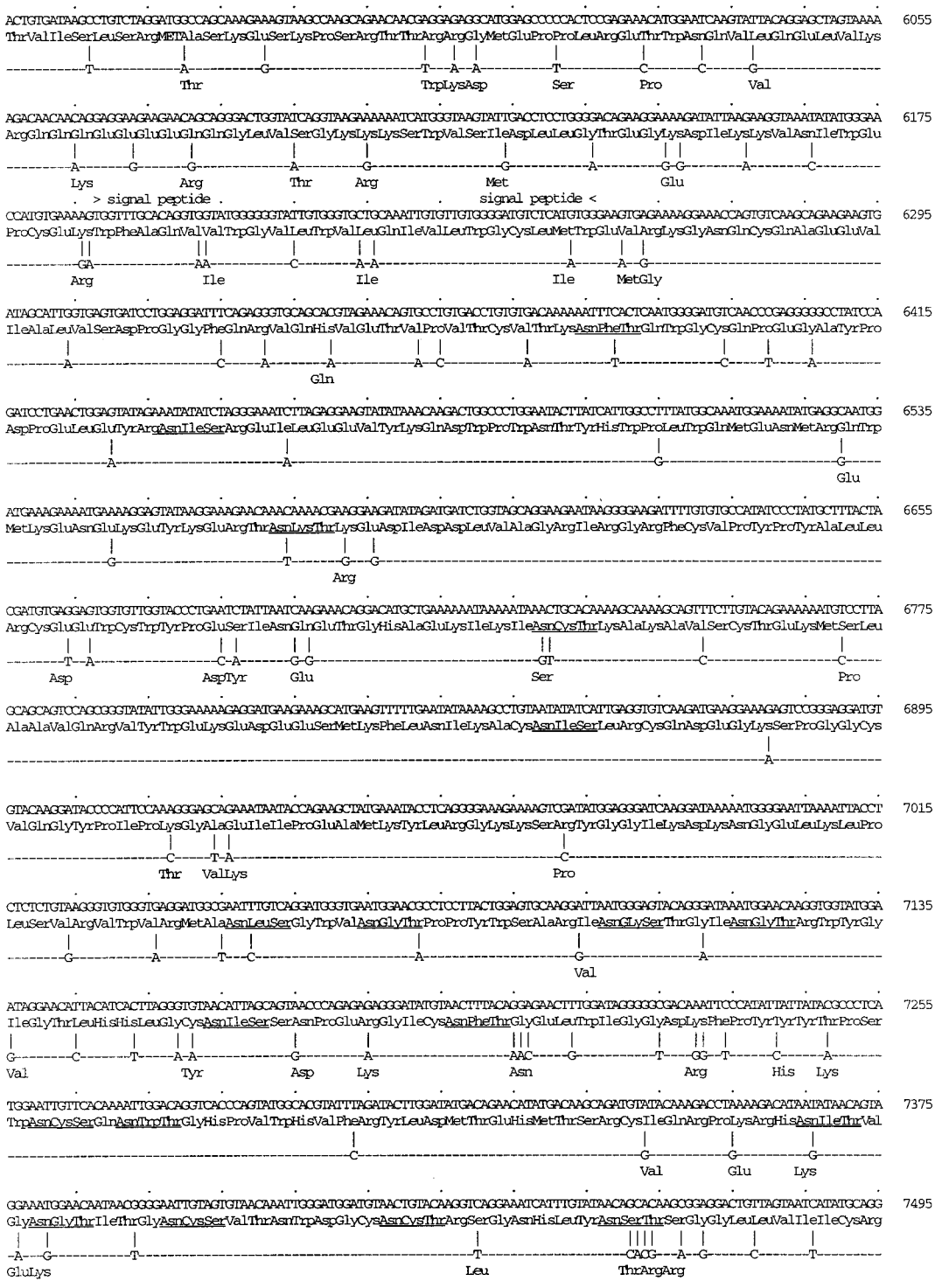
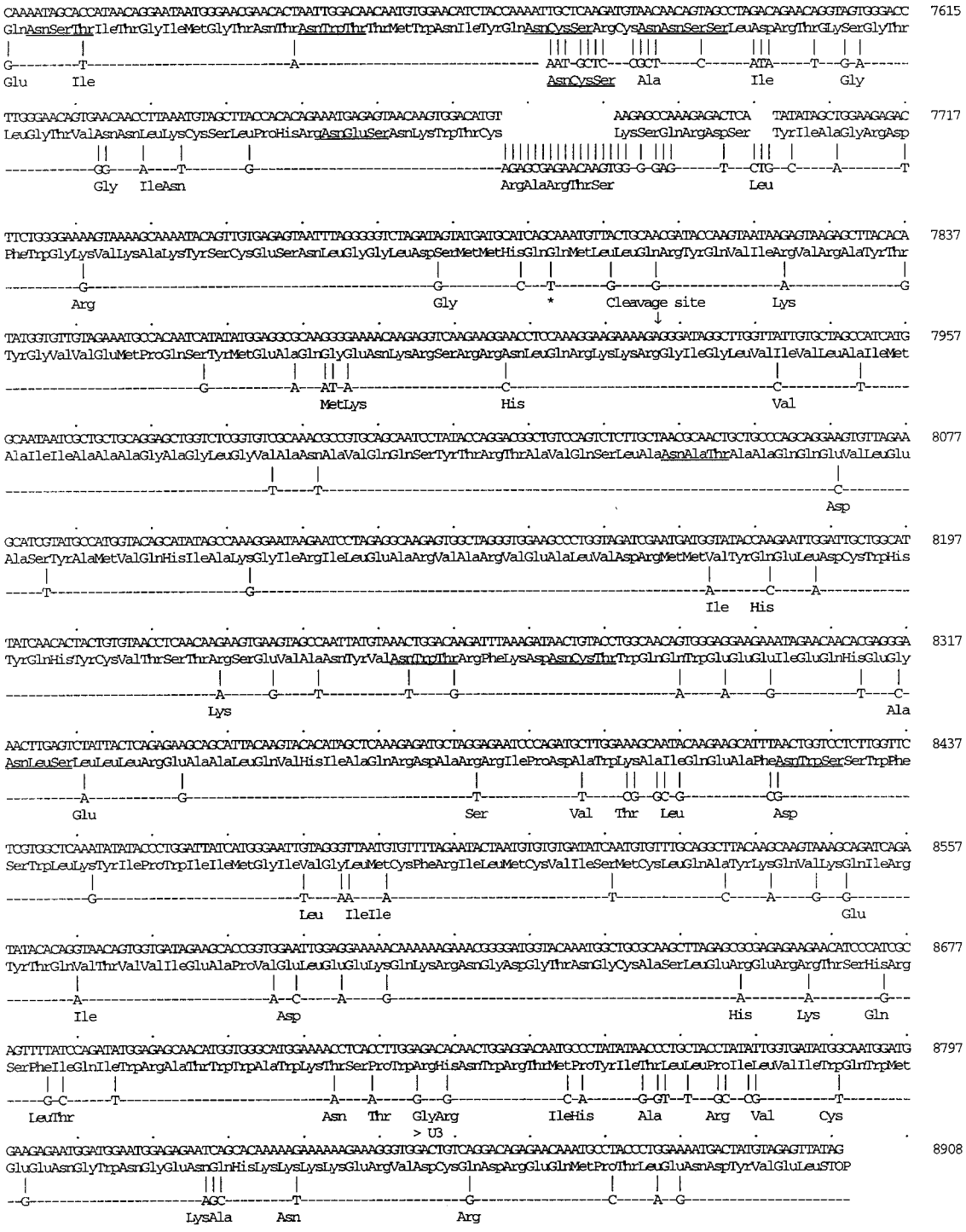


Fig. 3. Nucleotide sequence of the env gene in the maedi KM1071 strain compared to the visna KV1772 strain. The fully printed sequence (and numbering) is that of KV1772. Sequence identity is represented by dashes, and nucleotide changes and deduced amino acids are indicated. Potential glycosylation sites are underlined. A stop codon created by a transition at nt position 7790 is indicated (*).



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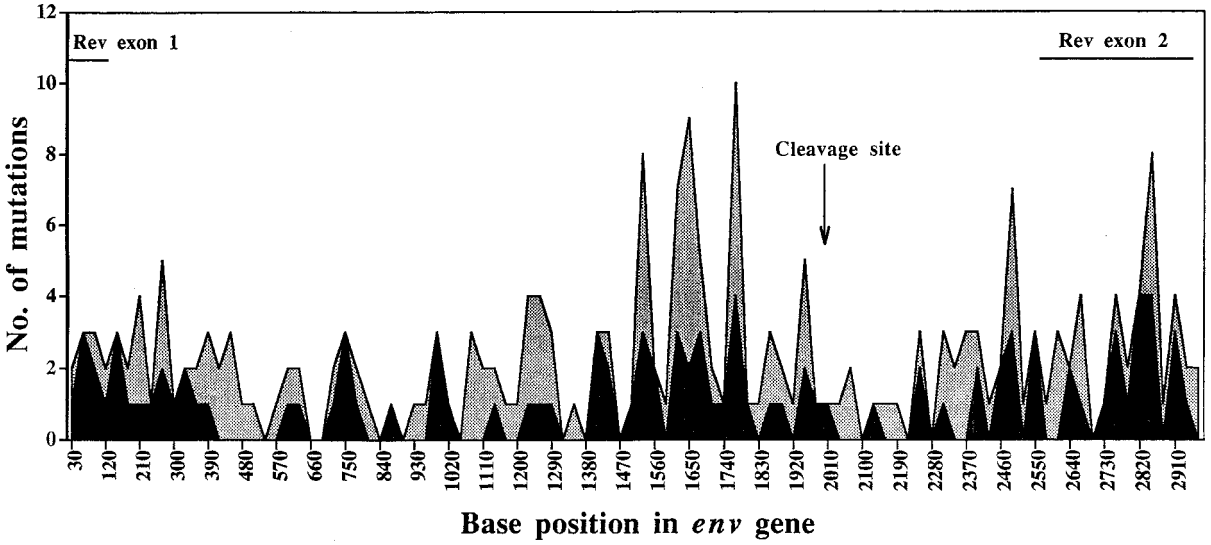

Fig. 4. Differences between maedi KM1071 and visna KV1772 virus envelope genes. The number of nucleic acid changes in 30 nucleotides were counted. The shaded area represents silent mutations and the black area represents nonsynonymous changes. Deletions are not counted.

the difference in neurovirulence. However, cumulative results with 10 intracerebral inoculations with the visna strains KV1514 and KV1772 showed that the average lesion grade was 3.5 and never below 2. This agrees with the results for visna virus presented in this paper and strengthens the indication that there is a real difference in neurovirulence between the visna and maedi strains. Infections with the molecularly cloned visna virus result in brain lesions of similar severity as with the uncloned visna virus (21).

Our findings of a higher growth rate of the visna virus strain KV1514 than the maedi strain KM1071 in SCP cells is in accord with earlier reports on *in vitro* behavior of other maedi and visna strains (4) and is reflected in the fact that end-point titration of maedi virus strains in SCP cells usually takes three weeks whereas it takes two weeks with visna virus strains. The possibility cannot be excluded that this difference is due to adaptation of the visna virus strains to SCP cells. It is, however, of interest in this context, that KV796, a common ancestor of the visna virus strains in this study, was originally isolated from choroid plexus (1) . The choroid plexus may be the initial site of entry for the neurotropic strains of MVV as has been suggested for infection with HIV-1 (29). This is supported by a high frequency of virus isolations from the choroid plexus in a large group of sheep infected with KV1514 (25), the density of antigen positive

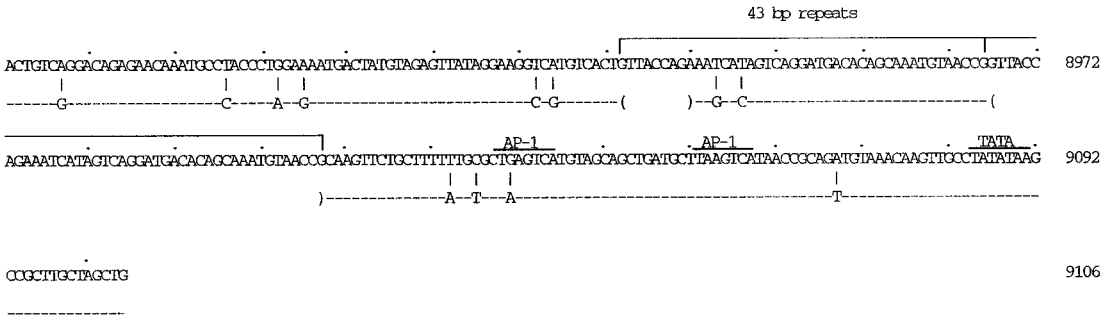

Fig. 5. Nucleotide sequence of the U3 region of the LTR in the maedi KM1071 and visna KV1772. The fully printed sequence (and numbering) is that of KV1772. Sequence identities are represented by dashes and deletions are indicated by parentheses.

cells in choroid plexus (9) and the initial CNS lesions, i.e. inflammation of the choroid plexus and of the subependymal region (2).

The molecularly cloned visna virus KV1772 kv72/67 retained the high growth rate of the visna strains in SCP cells and will therefore be a useful tool to map viral genetic determinants of the different cell tropism of maedi- and visna virus strains.

In macrophages, however, the growth rate was similar for the three strains. Thus the difference in neurovirulence in the MVV strains that we describe in this paper, which is quantitative rather than qualitative, is apparently not a special feature of macrophage-tropic MVV strains, in contrast to findings in other lentiviruses, i.e. HIV-1 (30) and SIV (31,32).

Sequencing of the *env* and LTR genes of the maedi strain KM1071 revealed an 11.7% difference in the Env protein when compared with known sequences of the visna strains KV1514 and KV1772 (33,34,21). A highly variable region was identified near the carboxyl end of the outer glycoprotein. This region includes a stretch of approximately 25 amino acids which lies between conserved cysteine residues and is largely hydrophilic (35) and may be analogous to the V3 loop of HIV (36).

Stop codons in the *env* gene seem to appear at a higher frequency in MVV than would be expected by chance. The first published sequence of MVV contains a stop codon in the *env* gene (33), and of four clones that have been sequenced of MVV strain KV1772, two contained stop codons in the *env* gene, and in one of the clones the stop codon occurred at the same site as we found in the maedi strain KM1071. These termination codons occur either in the last part of the outer glycoprotein or in the transmembrane protein. Sequencing of PCR products directly from tissue samples from infected sheep indicate that these premature stop codons are not as common *in vivo* as *in vitro* (data not shown). Stop codons have also been found in the *env* genes in SIV and HIV-2 (37,38). In these cases the stop codons occurred in the cytoplasmic domain of the transmembrane glycoprotein and were believed to arise in *in vitro* cultures.

We found considerable heterogeneity in the U3 of the LTR in the MVV strains as has been reported by others as well (39). There are several potential AP-1 binding sites in the MVV U3 region. The AP1 site most proximal to the TATA box has been shown to be active in transcription in the visna strain KV1514 (40). In maedi strain KM1071 this AP1 motif is imperfect, and this holds true also for other MVV strains that have been sequenced (28,41). In the visna strains, however, either of the two potential AP 1 sites proximal to the TATA box contain a perfect AP 1 motif. This may result in a higher rate of replication in the visna strains than in the maedi strains. Sargan et al. examined a number of LTR variants from the EV1 isolate for transcriptional activity in SCP cells and included the visna strain KV1514 described here. A higher rate of transcription was consistently observed from the KV1514 LTR than from the LTR of the different EV1 isolates (39).

We cannot deduce from the nucleotide sequence where in the genome the difference in neurovirulence and cell tropism resides, but further studies involving construction of chimeric viruses using the pathogenic molecular clone of visna virus are in progress.

Acknowledgments

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