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The *vif* gene of maedi-visna virus is essential for infectivity in vivo and in vitro

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Abstract

We have investigated the role of *vif* in maedi-visna virus (MVV), a lentivirus of sheep, by studying in vitro replication of *vif*-deleted MVV in several cell types, and the effects of *vif* deletion on in vivo infection. By measuring RT activity, we found that in comparison to wild-type MVV, growth of *vif*-deleted MVV was similar in fetal ovine synovial (FOS) cells, highly attenuated in sheep choroid plexus (SCP) cells, and not detectable in macrophages, natural target cells of MVV. Productive infection by *vif*-deleted MVV could not be demonstrated in sheep. An increased mutation frequency was observed in DNA produced by endogenous reverse transcription of viral RNA in *vif*-deleted virions, indicating the existence of a factor comparable in action to human APOBEC3G. These results suggest that the *vif* gene of MVV is essential for infectivity and that the Vif protein protects the viral genome from enpackaged mutagenic activities.

Keywords: Retroviruses; Lentiviruses; Vif; Maedi-visna virus; Infectivity; APOBEC3G; Cytidine deamination

Introduction

Maedi-visna virus (MVV), a member of the lentivirus subfamily, causes slowly progressive meningoencephalomyelitis (visna) and pneumonia (maedi) of sheep (Georgsson, 1990). MVV predominantly infects cells of the monocyte–macrophage lineage (Gendelman et al., 1986; Narayan et al., 1982). In addition to the structural genes *gag*, *pol* and *env*, MVV has two genes encoding the regulatory proteins Tat and Rev and an accessory gene *vif* (Sonigo et al., 1985), present in all known lentiviruses except equine infectious anemia virus (EIAV) (Kawakami et al., 1987; Oberste and Gonda, 1992). *Vif* is the only accessory gene conserved in the small ruminant lentiviruses MVV and caprine arthritis encephalitis virus (CAEV) (Pyper et al., 1986; Saltarelli et al., 1990; Sonigo et al., 1985). Sequence similarities among Vif proteins are low apart from a single highly conserved motif (S/T)LQ(F/Y/R)LA (Oberste and Gonda, 1992). Nevertheless, in human immunodeficiency virus type 1 (HIV-1), deletion of any region of five or six amino acids in Vif, except in the carboxy terminus, seems to disrupt the function (Simon et al., 1999). The requirement for Vif is cell-type dependent. When infected with *vif*-deleted viruses, cells nonpermissive for these deficient viruses, such as peripheral blood mononuclear cells (PBMCs), produce virions with impaired replication in any target cell, whereas *vif*-deleted viruses from permissive cell types replicate without hindrance (Courcoul et al., 1995; Fan and Peden, 1992; Fisher et al., 1987; Gabuzda et al., 1992; Sodroski et al., 1986; Strebel et al., 1987). Vif enhances virion infectivity in a poorly understood manner (Borman et al., 1995; Sakai et al., 1993; Simon and Malim, 1996; Sova and Volsky, 1993; Von Schwedler et al., 1993). HIV-1 lacking Vif has been reported to be up to 1000-fold less infective than wild type (Fisher et al., 1987; Kishi et al., 1992; Strebel et al., 1987) and the Vif protein is essential in HIV-1 and HIV type 2 (HIV-2) (Chowdhury et al., 1996; Courcoul et al., 1995; Fan and Peden, 1992; Gabuzda et al., 1992, 1994; Gibbs et al., 1994a; Kawamura et al., 1994; Michaels et al., 1993; Reddy et al., 1995), simian immunodeficiency virus (SIV) (Gibbs et al., 1994b; Park et al.,

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1994), feline immunodeficiency virus (FIV) (Lockridge et al., 1999; Tomonaga et al., 1992) and CAEV (Harmache et al., 1995) for productive infection of cultured primary cells. In vivo experiments with CAEV (Harmache et al., 1996), FIV (Inoshima et al., 1996, 1998; Lockridge et al., 2000) and SIV (Desrosiers et al., 1998) have confirmed the requirement for a functional *vif* gene for effective host infections, with delta-*vif* infections resulting in low viral loads and very weak antibody responses. New investigations have shown a requirement for HIV-1 Vif protein to counter G-to-A mutations in nascent retroviral DNA induced by the APOBEC3G protein (also know as CEM15) expressed in nonpermissive human cells (Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Sheehy et al., 2002; Zhang et al., 2003).

The aim of this study was to determine the importance of the MVV *vif* gene in vitro by infecting several cell-types with *vif*-deleted virus particles produced by sheep choroid plexus (SCP) cells, and in vivo by intratracheal inoculation of sheep with the same virus. In light of recent investigations, we have also determined the nature and frequency of mutations in Vif-deficient virions.

Results

Construction and identification of vif mutant viruses

The dVif-1 mutant used in this study carries a 447-bp in-frame deletion within the *vif* gene of MVV, obtained by *Sty*I digestion (Fig. 1). This mutant lacks about two-thirds of the wild-type Vif sequence. The cloned viral DNA was transfected into fetal ovine synovial (FOS) cells and the viral supernatants harvested at peak virus production and titered in SCP cells for stocks of dVif-1 viruses. The presence of the deletion in virus stocks was confirmed by PCR and sequencing.

MVV vif gene is necessary for efficient viral replication in cell culture

To determine the requirement for Vif in vitro, SCP grown cell-free titered preparations of dVif-1 and the wild-type virus KV1772 were used to infect FOS cells, SCP cells and macrophages. The replication potential of dVif-1 MVV was tested by daily monitoring of RT activity in supernatants harvested from infected cells and comparing it to wt MVV infections. In addition, cell cultures were inspected for cell damage under the microscope. As shown in Fig. 2, all three cell types allowed efficient replication of the wt virus KV1772. Growth of dVif-1 could not be detected by RT assay of infected macrophages (Fig. 2A). MVV Vif deficiency resulted in poor growth in SCP cells (Fig. 2B) with RT activity about 10-fold lower than with wt MVV. The dVif-1 virus replicated in FOS cells (Fig. 2C). Microscopic observations were consistent with these results, both dVif-1-infected SCP cells and macrophages exhibited very little cell damage comparable to uninfected cells, while cells infected with wt virus showed severe cytopathic effects. dVif-1 virus growth on FOS cells was comparable to wt virus, but cytopathic effects were delayed by several days. These data suggest that the Vif protein also plays an important part during in vivo infections with MVV. To test this hypothesis, we studied the effect of dVif-1 infections in sheep.

A.

```
      63                                                  219
      Lys Glu Asn Gln  Gly              Ile  Pro Trp Ser Leu
      AAA GAA AAC CAA G/GG...        ...ATA C/CT TGG TCT CTG
      5151       StyI                      StyI      5619
```

B.

```
          1
Vif wt  MLSSYRHQKK YKKNKAREIG PQLPLWAWKE TAFSINQEPY WYSTIRLQGL
dVif-1  MLSSYRHQKK YKKNKAREIG PQLPLWAWKE TAFSINQEPY WYSTIRLQGL
         51
Vif wt  MWNKRGHKLM FVKENQGYEY WETSGKQWKM EIRRDLDLIA QINFRNAWQY
dVif-1  MWNKRGHKLM FVKENQ---- ---------- ---------- ----------
        101
Vif wt  KSQGEWKTIG VWYESPGDYK GKENQFWFHW RIALCSCNKT RWDIREFMIG
dVif-1  ---------- ---------- ---------- ---------- ----------
        151
Vif wt  KHRWDLCKSC IQGEIVKNTN PRSLQRLALL HLAKDHVFQV MPLWRARRVT
dVif-1  ---------- ---------- ---------- ---------- ----------
        201
Vif wt  VQKFPWCRSP MGYTIPWSLQ ECWEMESIFE
dVif-1  ---------- ------WSLQ ECWEMESIFE
```

Fig. 1. Construction of dVif-1 mutant. (A) Staggered ends generated by *Sty*I digestion were filled in and ligated. (B) Deduced amino acid sequences of the Vif protein encoded by the wild-type MVV KV1772 and the dVif-1 mutant, with broken line indicating deletion.

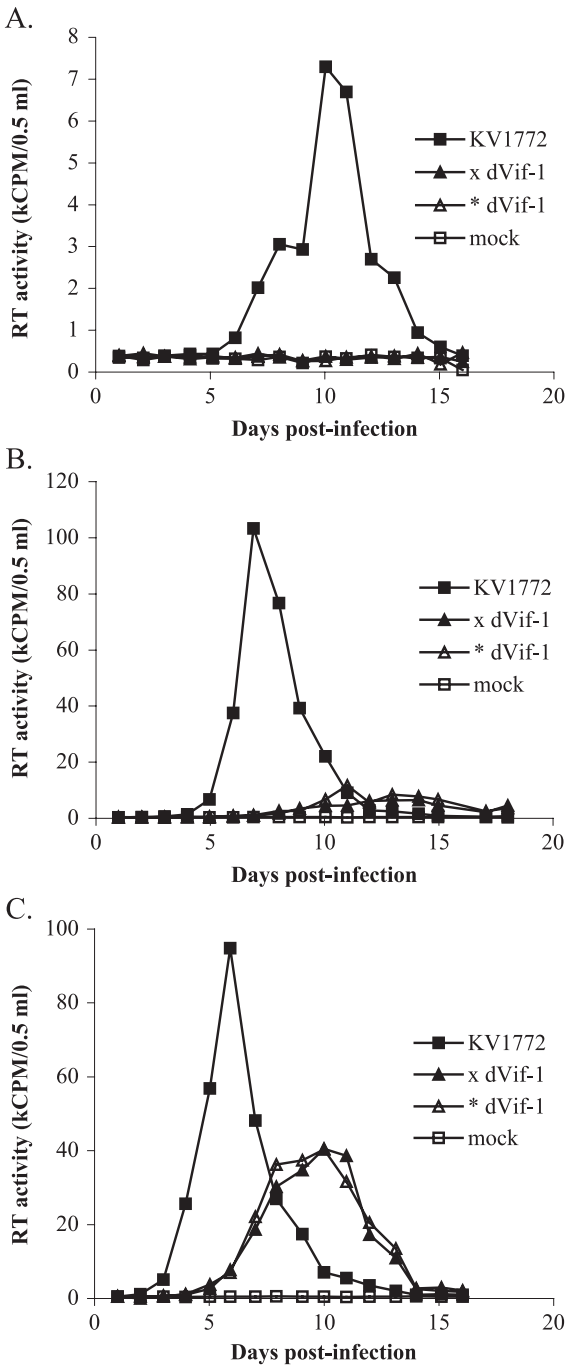

Fig. 2. Analysis of dVif-1 mutant virus replication as compared to wild-type KV1772 in macrophages (A), SCP cells (B) and FOS cells (C) following cell-free infection. The multiplicity of infection (MOI) was 0.1. Two independent stocks of the dVif-1 mutant were used, represented as x dVif-1 and * dVif-1. Uninfected cells were used as negative control. Samples were taken from cell culture medium, virions pelleted by centrifugation and RT activity measured.

MVV vif gene is required for efficient replication in vivo

To assess the ability of dVif-1 to produce in vivo infection, three sheep (animals 2083, 2084 and 2085) were inoculated intratracheally with dVif-1 virus. Three control sheep (animals 2080, 2081 and 2082) were inoculated with the wild-type virus KV1772. Blood samples were taken before infection, at weeks 2, 3, 4, 5, 7 and 8 after infection and then every other week until sacrifice at 18 weeks. The blood samples were assessed for virus presence and antibodies to MVV proteins. Neurovirulence was assessed by analyzing samples of cerebrospinal fluid for cells and virus. Several organs were assayed for virus presence. The virus could not be isolated from any of the dVif-1 infected sheep, neither from blood nor any of the organs tested, whereas KV1772-inoculated sheep showed multiorgan infections. The virus was frequently isolated from blood during the observation period in all sheep infected with wt virus but never in dVif-1-infected sheep (Table 1). SCP cells are routinely used in our laboratory for propagating MVV including co-culturing infected organs. However, because FOS cells were more permissive for Vif-deficient MVV than SCP cells, we also used FOS cells for attempting virus isolation from organs. At sacrifice, the virus was isolated from the lungs and all lymphoid organs tested from control sheep 2080 and 2081 and from spleen and bone marrow of sheep 2082. In sheep 2080, the virus was recovered from the cerebrum. In repeated tests, the two dVif-1-infected sheep 2083 and 2084 showed weak antibody responses to MVV in ELISA analysis of sera taken 16 and 18 weeks postinfection (respectively), giving an indication of infection (Fig. 3). These two sheep also had a faint reaction on gag-p25 viral antigens in Western blots (data not shown). The dVif-1-inoculated sheep 2085 was negative both in ELISA and Western blots. The KV1772-infected controls developed strong antibody responses as shown by ELISA (Fig. 3) and in Western blots (data not shown).

Increased mutation frequency in Vif-deficient virions

To assess the spectrum and frequency of mutations in dVif-1 virions, endogenous DNA synthesis was induced in pelleted virions and a section corresponding to the capsid protein was amplified, cloned and sequenced. Analysis of sequences from dVif-1 virions grown in FOS and SCP cells

Table 1
Frequency of virus isolation from KV1772 and dVif-1 infected sheep

	Blood[a]	CNS and CSF[b]	Lymphoid tissue[b,c]	Lungs[b]
KV1772				
2080	10/11	1/4	5/5	2/2
2081	8/11	0/4	5/5	2/2
2082	5/11	0/4	2/5	0/2
dVif-1				
2083	0/11	0/4	0/5	0/2
2084	0/11	0/4	0/5	0/2
2085	0/11	0/4	0/5	0/2

[a] SCP cells were used for virus culture.
[b] FOS and SCP cells were used for virus culture.
[c] Spleen, bone marrow, cervical, mediastinal, and mesenteric lymph nodes.

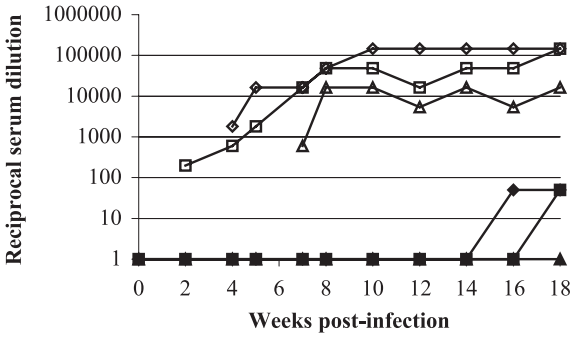

Fig. 3. Time course of antibody response measured by ELISA. Sheep infected with wild-type MVV KV1772, open labels; 2080 (◇), 2081 (□) and 2082 (△), and dVif-1 infected, closed labels; 2083 (◆), 2084 (■) and 2085 (▲). Serum dilution series were incubated with immobilized MVV antigens and endpoints determined ($A_{492} \leq 0.4$).

showed a greatly increased mutation frequency compared to KV1772 (wt) grown in SCP cells (Fig. 4). G-to-A transitions corresponding to cytidine deamination of the first strand DNA were most common (13/18) as observed with Vif-deficient HIV-1 virions (Lecossier et al., 2003; Mangeat et al., 2003; Zhang et al., 2003). Four out of the 18 mutations were A-to-G transitions.

Discussion

In this report, we demonstrate the importance of the *vif* gene for MVV infectivity in vitro and in vivo. The auxiliary gene *vif*, encoded by all known lentiviruses except EIAV, has been reported to increase HIV-1 infectivity by up to 1000-fold (Fisher et al., 1987; Kishi et al., 1992; Strebel et

A. KV1772 (wt) in SCP

```
              E  S  V   V  F  Q   Q  L  Q  T   V  A  M   Q  H  G   L  V  S  E
961       tagagtcagt agtcttccag caactgcaaa cagtggcaat gcagcatgga cttgtgtccg
1x(wt1)   .......... .......... .......... .......... .......... ..........
1x(wt2)   .......... .......... .......... .......... .......... ..........
9x        .......... .......... .......... .......... .......... ..........

              D  F  E   R  Q  L   A  Y  Y  A   T  T  W   T  S  K   D  I  L  E
1021      aggattttga gaggcaattg gcatattatg ctactacctg gactagtaaa gatatattag
1x(wt1)   .......... .......... .......... .......... .......... ..........
1x(wt2)   .......... .......... .......... .......... .......... ..........
9x        .......... .......... .......... .......... .......... ..........

              V  L  A   M  M  P   G  N  R  A   Q  K  E   L  I  Q   G  K  L  N
1081      aagtattggc tatgatgcct gggaatagag cacagaagga attaatacaa ggaaaattaa
1x(wt1)   .......... .......... .......... .......... .......... ..........
1x(wt2)   .......... .......... .......... .......... .......... ..........
9x        .......... .......... .......... .......... .......... ..........

              E  E  A   E  R  W   V  R  Q  N   P  P  G   P  N  V   L  T  V  D
1141      atgaagaagc agaaaggtgg gtaagacaaa atccacccgg gccgaatgtc ctcacggtgg
1x(wt1)   .......... .......... .......... .......... .......... ..........
1x(wt2)   .......... .......... .......... .......... .......... ..........
9x        .......... .......... .......... .......... .......... ..........
              Q  I  M   G  V  G   Q  T  N  Q   Q  A  S   Q  A  N   M  D  Q  G
1201      atcaaataat gggagtggga caaaccaatc agcaggcatc tcaagccaat atggatcagg
1x(wt1)   .......... .......... .......... .......... .......... ..........
1x(wt2)   .......... .......... .......... .......... .......... ...a......
9x        .......... .......... .......... .......... .......... ..........

              R  Q  I   C  L  Q   W  V  I  T   A  L  R   S  V  R   H  M  S  H
                                    R
1261      caagacagat atgcctgcag tgggtaataa cagcgttaag atcagtgagg catatgtcac
1x(wt1)   .......... .......... .......... .......... .......... ..........
1x(wt2)   .......... .......... .......g.. .......... .......... ..........
9x        .......... .......... .......... .......... .......... ..........

              R  P  G   N  P  M   L  V  K  Q   K  N  T   E  S  Y   E  D  F  I
1321      atagaccagg aaaccctatg ttagtgaagc agaagaatac tgagagttat gaagacttca
1x(wt1)   .......... .......... .......... .......... .......... ..........
1x(wt2)   .......... .......... .......... .......... .......... ..........
9x        .......... .......... .......... .......... .......... ..........
```

Fig. 4. Endogenous viral RT products were amplified, cloned and sequenced. Sequences from nucleotide position 961–1380 of the capsid segment of MVV were determined. (A) KV1772 (wt) virus passaged eight times in SCP cells. (B) dVif-1 virus passaged once in FOS cells. (C) dVif-1 virus passaged once in SCP cells. The sequence of the KV1772 (wt) DNA clone is shown at the top of each section and the number of clones of each sequence is indicated (1×, 7×, . . .). The amino acid sequence and substitutions resulting from mutations are shown above the nucleotide sequences.

B. dVif-1 in FOS

```
              E  S  V   V  F  Q   Q  L  Q  T   V  A  M   Q  H  G   L  V  S  E
961           tagagtcagt agtcttccag caactgcaaa cagtggcaat gcagcatgga cttgtgtccg
1x(F2)        .......... .......... .......... .......... .......... ..........
1x(F6)        .......... .......... .......... .......... .......... ..........
1x(F8)        .......... .......... .......... .......... .......... ..........
1x(F9)        .......... .......... .......... .......... .......... ..........
1x(F10)       .......... .......... .......... .......... .......... ..........
1x(F12)       .......... .......... .......... .......... .......... ..........
7x            .......... .......... .......... .......... .......... ..........

              D  F  E   R  Q  L   A  Y  Y  A   T  T  W   T  S  K   D  I  L  E
1021          aggattttga gaggcaattg gcatattatg ctactacctg gactagtaaa gatatattag
1x(F2)        .......... .......... .......... .......... .......... ..........
1x(F6)        .......... .......... .......... .......... .......... ..........
1x(F8)        .......... .......... .......... .......... .......... ..........
1x(F9)        .......... .......... .......... .......... .......... ..........
1x(F10)       .......... .......... .......... .......... .......... ..........
1x(F12)       .......... .......... .......... .......... .......... ..........
7x            .......... .......... .......... .......... .......... ..........

              V  L  A   M  M  P   G  N  R  A   Q  K  E   L  I  Q   G  K  L  N
1081          aagtattggc tatgatgcct gggaatagag cacagaagga attaatacaa ggaaaattaa
1x(F2)        .......... .......... .......... .......... .......... ..........
1x(F6)        .......... .......... .......... .g........ .......... ..........
1x(F8)        .......... .......... .......... .......... .......... ..........
1x(F9)        .......... .......... .......... .......... .......... ..........
1x(F10)       .......... .......... .......... .......... .......... ..........
1x(F12)       .......... .......... .......... .......... .......... ..........
7x            .......... .......... .......... .......... .......... ..........

              E  E  A   E  R  W   V  R  Q  N   P  P  G   P  N  V   L  T  V  D
1141          atgaagaagc agaaaggtgg gtaagacaaa atccacccgg gccgaatgtc ctcacggtgg
1x(F2)        .......... .......... .......... .......... .......... ..........
1x(F6)        .......... .......... .......... .......... .......... ..........
1x(F8)        .......... .......... .......... .......... .......... ..........
1x(F9)        .......... .......... .......... .......... .......... ..........
1x(F10)       .......... .......... .......... .......... .......... ..........
1x(F12)       .......... .......... .......... .......... .......... ..........
7x            .......... .......... .......... .......... .......... ..........

              Q  I  M   G  V  G   Q  T  N  Q   Q  A  S   Q  A  N   M  D  Q  G
1201          atcaaataat gggagtggga caaaccaatc agcaggcatc tcaagccaat atggatcagg
1x(F2)        .......... .......... .......... .......... .......... ..........
1x(F6)        .......... .......... .......... .......... .......... ..........
1x(F8)        .......... .......... .......... .......... .......... ..........
1x(F9)        .......... .......... .......... .......... .......... ..........
1x(F10)       .......... .......... .......... .......... .......... ..........
1x(F12)       .......... .......... .......... .......... .......... ..........
7x            .......... .......... .......... .......... .......... ..........

              R  Q  I   C  L  Q   W  V  I  T   A  L  R   S  V  R   H  M  S  H
                 R
1261          caagacagat atgcctgcag tgggtaataa cagcgttaag atcagtgagg catatgtcac
1x(F2)        .......... .......... .......... .......... .......... ..........
1x(F6)        .......... .......... .......... .......... .......... ..........
1x(F8)        ......g... .......... .......... .......g.. .......... ..........
1x(F9)        .......... .......... .......... .......... .......... ..........
1x(F10)       .......... .......... .......... .......... .......... ..........
1x(F12)       .......... .......... .......... .......... .......... ..........
7x            .......... .......... .......... .......... .......... ..........

              R  P  G   N  P  M   L  V  K  Q   K  N  T   E  S  Y   E  D  F  I
                                                                    K
1321          atagaccagg aaaccctatg ttagtgaagc agaagaatac tgagagttat gaagacttca
1x(F2)        .......... .......... .....a.... .a..a..... .......... ..........
1x(F6)        .......... .......... .......... .......... .......... ..........
1x(F8)        .......... .......... .......... .......... .......... ..........
1x(F9)        .......... .......... .....a.... .......... .......... ..........
1x(F10)       .......... .......... .......... .a..a..... .......... ..........
1x(F12)       .......... .......... .......... .......... .......... a.........
7x            .......... .......... .......... .......... .......... ..........
```

Fig. 4 (*continued*).

C. dVif-1 in SCP

```
              E  S  V   V  F  Q   Q  L  Q  T   V  A  M   Q  H  G   L  V  S  E
              K
961           tagagtcagt agtcttccag caactgcaaa cagtggcaat gcagcatgga cttgtgtccg
1x(P13)       .......... .......... .......... .......... .......... ..........
1x(P15)       .......... .......... .......... .......... .......... ..........
1x(P20)       ..a....... .......... .......... .......... .......... ..........
1x(P22)       .......... .......... .......... .......... .......... ..........
7x            .......... .......... .......... .......... .......... ..........

              D  F  E   R  Q  L   A  Y  Y  A   T  T  W   T  S  K   D  I  L  E
                                                                             K
1021          aggattttga gaggcaattg gcatattatg ctactacctg gactagtaaa gatatattag
1x(P13)       .......... .......... .......... .......... .......... .........a
1x(P15)       .......... .......... .......... .......... .......... ..........
1x(P20)       .......... .......... .......... .......... .......... ..........
1x(P22)       .......... .......... .......... .......... .......... ..........
7x            .......... .......... .......... .......... .......... ..........

              V  L  A   M  M  P   G  N  R  A   Q  K  E   L  I  Q   G  K  L  N
                                                                    R
1081          aagtattggc tatgatgcct gggaatagag cacagaagga attaatacaa ggaaaattaa
1x(P13)       .......... .......... .......... .......... .......... ..........
1x(P15)       .......... .......... .......... .......... .......... ..........
1x(P20)       .......... .......... .......... .......... .......... a.........
1x(P22)       .......... .......... .......... .......... .......... ..........
7x            .......... .......... .......... .......... .......... ..........

              E  E  A   E  R  W   V  R  Q  N   P  P  G   P  N  V   L  T  V  D
1141          atgaagaagc agaaaggtgg gtaagacaaa atccacccgg gccgaatgtc ctcacggtgg
1x(P13)       .......... .......... .......... .......... .......... ..........
1x(P15)       .......... .......... .......... .......... .......... ..........
1x(P20)       .......... .......... .......... .......... .......... ..........
1x(P22)       .......... .......... ..g....... .......... .......... ..........
7x            .......... .......... .......... .......... .......... ..........

              Q  I  M   G  V  G   Q  T  N  Q   Q  A  S   Q  A  N   M  D  Q  G
1201          atcaaataat gggagtggga caaaccaatc agcaggcatc tcaagccaat atggatcagg
1x(P13)       .......... .......... .......... .......... .......... ..........
1x(P15)       .......... .......... .......... .......... .......... ..........
1x(P20)       .......... .......... .......... .......... .......... ..........
1x(P22)       .......... .......... .......... .......... .......... ..........
7x            .......... .......... .......... .......... .......... ..........

              R  Q  I   C  L  Q   W  V  I  T   A  L  R   S  V  R   H  M  S  H
1261          caagacagat atgcctgcag tgggtaataa cagcgttaag atcagtgagg catatgtcac
1x(P13)       .......... .......... .......... .......... .......... ..........
1x(P15)       .......... .......... .......... .......... .......... ..........
1x(P20)       .......... .......... .......... .......... .......... ..........
1x(P22)       .......... .......... .......... .......... .......... ..........
7x            .......... .......... .......... .......... .......... ..........

              R  P  G   N  P  M   L  V  K  Q   K  N  T   E  S  Y   E  D  F  I
1321          atagaccagg aaaccctatg ttagtgaagc agaagaatac tgagagttat gaagacttca
1x(P13)       .......... .......... .......... .......... .......... ..........
1x(P15)       .......... .......... .......... ....a..... .......... ..........
1x(P20)       .......... .......... .....a.... ....a..... .......... ..........
1x(P22)       .c........ .......... .......... .......... .......... ..........
7x            .......... .......... .......... .......... .......... ..........
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Fig. 4 (*continued*).

al., 1987). Despite extensive studies, the basis of Vif function is still unclear. We constructed a *vif*-deleted MVV mutant with an in-frame deletion of about two-thirds of the *vif* gene in the infectious MVV strain KV1772 (wt). Using RT assays to compare the replication properties of *vif*-deleted mutant to wt viruses, viral production could not be detected in cultured macrophages infected with the mutant virus, whereas wt viruses were produced in macrophages (Fig. 2A). Macrophages are considered natural target cells of MVV infection (Gendelman et al., 1986; Narayan et al., 1982). These results are consistent with previous reports showing that the active Vif protein was crucial for HIV-1 and HIV-2 infections of cultured primary cells (Chowdhury et al., 1996; Courcoul et al., 1995; Fan and Peden, 1992; Gabuzda et al., 1992, 1994; Gibbs et al., 1994a; Kawamura et al., 1994; Michaels et al., 1993; Reddy et al., 1995), as

well as for SIV (Gibbs et al., 1994b; Park et al., 1994), FIV (Lockridge et al., 1999; Tomonaga et al., 1992) and CAEV (Harmache et al., 1995). Besides macrophages, we tested the replication of *vif*-deleted MVV in two other cell-types, SCP and FOS cells. The growth of dVif-1 was highly attenuated in SCP cells but not in FOS cells (Figs. 2B–C). The replication properties of *vif*-deleted HIV-1 have been described for wide ranges of cell types and they have been categorized as permissive if replication is not affected by the Vif deficiency, or nonpermissive, if noninfectious virions are produced by the cells. This suggests that non-permissive cells produce a cellular factor or factors with antiviral activity, overcome by Vif action, or the presence of a positive factor in permissive cells, compensating Vif loss. Heterokaryons of permissive and nonpermissive cells show a nonpermissive phenotype, indicating the existence of an endogenous inhibitor of HIV (Madani and Kabat, 1998; Simon et al., 1998a). Ovine cells differ with respect to the degree of growth of *vif*-deleted MVV, as exemplified by FOS cells, which can be categorized as semipermissive and nonpermissive SCP cells and macrophages. A possible explanation could be the different expression of a cellular factor interacting with Vif. Recently, a protein produced by human nonpermissive cells but not permissive cells, APOBEC3G (CEM15), was reported to convert a permissive phenotype to a nonpermissive phenotype when ectopically expressed in permissive cells (Sheehy et al., 2002). APOBEC3G triggers substantial G-to-A mutations in the nascent DNA in the absence of Vif (Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Zhang et al., 2003).

Another example of a protein specific for nonpermissive cells is a nuclear body protein, Sp140, reported to bind to HIV-1 Vif. HIV-1 infection induced partial dispersal of the protein from nuclear bodies into the cytosol where it appeared to colocalize with Vif (Madani et al., 2002). Stable expression of Sp140 in a permissive cell line was not accomplished, but HeLa cells transiently expressing the protein did not convert to a nonpermissive phenotype. It has been reported that primate lentiviral Vif proteins function only in lymphocytes of species that are closely related (Simon et al., 1998b). Thus, it is possible that Vif interacts with a cellular factor that has changed during evolution of different hosts. Thus, although Vif derived from HIV-1 counteracts human APOBEC3G action in a broad range of retroviruses (Harris et al., 2003; Mangeat et al., 2003), this does not hold for APOBEC3G from mouse, African Green Monkey or rhesus macaque (Mariani et al., 2003).

Intratracheal inoculation of sheep with *vif*-deleted viruses resulted in weak antibody responses as detected by ELISA (Fig. 3). Inoculation of wt MVV resulted in multiorgan infections, whereas sheep inoculated with *vif*-deleted MVV showed no detectable viral infection in any of the tested organs during the time course of the experiment. These results demonstrate that the *vif* gene of MVV is essential for virus infectivity in vivo. Previous reports have shown the requirement for a functional *vif* gene for effective host infections by CAEV (Harmache et al., 1996), FIV (Inoshima et al., 1996, 1998; Lockridge et al., 2000) and SIV (Desrosiers et al., 1998), and reports indicate a role for Vif in the progression of AIDS (Alexander et al., 2002; Hassaine et al., 2000).

Analysis of DNA produced by endogenous reverse transcription in dVif-1 virions provides support for the presence of a cytidine deaminase activity analogous to the APOBEC3G protein found in Vif-deficient HIV-1 virions (Fig. 4), although the cytidine deaminase activity in MVV dVif-1 grown in FOS and SCP cells appears to be 10-fold lower than in HIV-1 grown in nonpermissive cells (Lecossier et al., 2003). The observed mutation frequency of approximately 0.1% in dVif-1 corresponds to nine mutations per genome and may therefore contribute to lower viability and infectivity. Although SCP cells are more restrictive for growth of dVif-1 virus than FOS cells, no difference in mutation frequency was observed. If this holds true for all the viral genome, the substantial differences in growth of dVif-1 viruses on SCP and FOS cells documented here must have other explanations than an increased frequency of mutations.

A notable difference in *vif*-deleted virions of MVV vs. HIV-1 is that A-to-G transitions constitute a larger fraction of the mutations, or 4/18 vs. 2/31 (Lecossier et al., 2003). Hypermutation of A-to-G has been observed in the analysis of spleen necrosis virus replication, suggesting a role for adenosine deaminase acting on double-stranded RNA (ADAR) in retroviral mutations (Kim et al., 1996). Further support for the role of adenosine deaminases and the involvement of secondary structures comes from analysis of A-to-G hypermutations in human respiratory syncytial virus (Martinez and Melero, 2002). A-to-G transitions in MVV and HIV-1 appear to be more dispersed than hypermutations described in spleen necrosis virus and human respiratory syncitial virus and may therefore reflect a different mechanism. It also remains to be determined whether the A-to-G mutations observed in Vif-deficient MVV and HIV-1 are due to a second enzymatic activity of APOBEC3G rather than a second enzyme such as an ADAR. It is possible that mammalian cells have developed a range of enzymes, including cytidine and adenosine deaminases, to destroy invading nucleic acids, and that some of the lentiviruses have acquired and developed an activity, the Vif protein, to counteract these defenses.

Materials and methods

Molecular clone and construction of mutant

The *vif*-deletion mutant clone, designated dVif-1, was constructed from the infectious wild-type molecular clone of MVV KV1772kv72/67 (KV1772) (Andresson et al., 1993). A plasmid subclone containing a DNA fragment from the *Bam*H1 site at 4587 to the *Hin*cII site at 6392 was digested

with restriction enzyme *Sty*I, filled in with DNA polymerase I Klenow fragment and the blunt ends ligated. This generated a 447-bp in-frame deletion (bases 5164–5610) within the *vif* gene. PCR using the forward primer 5′-CCATAAGGCTACAAGGGT TGA-3′ and the reverse primer 5′-TAGCCATCGTTGTAGTCTTTCG-3′ was used to confirm mutant constructs. The following labelled primers were used for sequencing *vif* in the ALFexpress (Pharmacia): 5′-Cy5-CATACCGCCACCAAAAGAAAT-3′ or 5′-Cy5-GGGCATCCAGGAGAGTGGCAAGGA-3′.

Cells, transfections and infections

FOS cells (Andresson et al., 1993) and sheep choroid plexus (SCP) cells (Sigurdsson et al., 1960) were grown and maintained in Dulbecco's minimal essential medium (GibcoBRL) supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin and either 10% lamb serum (growth medium) or 1% lamb serum (maintenance medium). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. PBMCs were isolated from healthy sheep by sedimentation of heparinized blood diluted with phosphate-buffered saline (PBS) on Histopaque-1077 (Sigma). PBMCs obtained were treated with lysing buffer (0.14 M NH_4Cl + 0.02 M Tris–HCl, pH 7.2) to lyse red blood cells, thoroughly washed with PBS and resuspended at 12×10^6 cells/ml in growth medium supplemented with 15% lamb serum and 5×10^{-5} M 2-mercaptoethanol. After 24 h, supernatant and unattached cells were removed and new growth medium added. Adherent macrophages were grown for 7 days before they were infected.

Transfection of FOS cells was performed with Lipofectamine as specified by the manufacturer (GibcoBRL). A day after transfection, the lamb serum content was decreased from 20% to 10% by replacing the growth medium and again from 10% to 2% after 4 days. Viral supernatants with maximum reverse transcription (RT) activity (harvested 21 days after transfection) were used to infect SCP cells, which are routinely used in our laboratory for propagating MVV. The dVif-1 clone grew to a $TCID_{50}$ titer of $10^{5.5}$/ml and virus stocks were stored at −80 °C. Infections were carried out at a multiplicity of infection (MOI) of 0.1 with adsorption for 4 h at 37 °C.

RT activity

Viral particles from 0.5 ml of cell-free supernatants from infected or transfected cells were pelleted by centrifugation at 4 °C for 1 h at 20,000 × *g*. RT activity was determined by using [^{3}H]TTP (30 Ci/mmol) as previously described (Turelli et al., 1996).

Mutations in endogenous DNA

Viral particles in 0.5 ml of cell-free supernatants were adjusted to 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 50 units of DNAse I added and incubated at 37 °C for 30 min. Viral pellets were resuspended in 40 μl of 50 mM Tris–HCl (pH 8.0), 4 mM $MgCl_2$, 0.125% NP40 and 0.5 mM dNTPs, incubated at 40 °C for 120 min and heated 5 min at 90 °C. The capsid segment of MVV was amplified with primers V919Eco: 5′-CCGGAATTCCCTATTGTGAATTTGCAAGC-3′ and V-1578Xba: 5′-GCTCTAGATTAAAATCCTTCGGATCCCAC-3′, 35 cycles of 94 °C for 30 s, 55 °C for 20 s and 72 °C for 30 s using a mixture of 0.4 units Taq (Amersham-Pharmacia) and 0.04 units Pfu (Stratagene) polymerases in a 40-μl reaction. The products were gel purified (Qiaex II from Qiagen), cloned in a Bluescript II SK vector and sequenced using BigDye cycle sequencing ver. 1.1 (Applied Biosystems) and an ABI 310 instrument.

Infections of sheep and tissue sampling

Icelandic sheep (8 months old) were infected by intratracheal inoculation of 1.0 ml containing 1×10^4 $TCID_{50}$ of the appropriate MVV strain. Virus was injected into the trachea with a needle (23G) under sedation with 0.2 ml xylazinum i.v. (Xylapan). Blood samples were taken before infection, at weeks 2, 3, 4, 5, 7 and 8 after infection and then every other week until sacrifice at 18 weeks. Virus isolation from buffy coat was performed by coculture of white blood cells and SCP cells as previously described (Torsteinsdottir et al., 1997) and serum samples were assayed for the presence of virus-specific antibodies. Samples of cerebrospinal fluid were collected at sacrifice for cell counts and coculture with SCP cells for virus isolation. The following tissues were assayed for virus by coculture with SCP (Petursson et al., 1976) and FOS cells: choroid plexus, cerebrum, cerebellum, spleen, cervical, mediastinal and mesenteric lymph nodes, bone marrow and lungs. Coculture with FOS cells, in 12-well plates, was observed for 2 weeks. If no cytopathic effects were observed, the growth medium was passaged twice, with the last observation after 6 weeks of culture.

ELISA analyses and Western blot

Visna virus antigen for ELISA was prepared as previously described (Torsteinsdottir et al., 1997). ELISA was performed according to a slightly modified method from Houwers et al. (1982) as described elsewhere (Torsteinsdottir et al., 1997). In brief, microtiter plates (Dynatech-Immunlon) were coated with 100 μl of 1/400 dilution of visna ELISA antigen. Threefold serum dilutions were made, starting with dilution 1/200. For dVif-1-infected sheep, 2-fold serum dilutions were also made, starting with dilution 1/50 and ending in 1/400. Unbound material was removed by washing with PBS containing 0.05% Tween 20. Rabbit anti-goat IgG conjugated to horseradish peroxidase (Sigma A4174) (1/4000 dilution) was applied, and bound antibody detected with H_2O_2/*o*-phenylenedi-

amine dihydrochloride (OPD) (Dako). After 30 min, 75 μl of 4 M H_2SO_4 per well was added and the absorbance at 492 nm read in a Perkin-Elmer HTSoft 7000Plus Bio Assay Reader. Western blot strips were prepared using concentrated visna virus as previously described (Torsteinsdottir et al., 1997). The strips were incubated with serum samples diluted 1/200 overnight on a roller at 4 °C, before washing in 0.1 M Tris–HCl-buffered saline, pH 7.8, containing 0.1% Tween 20 (TBS-T) for 5 min five times. Rabbit anti-goat IgG conjugated to alkaline phosphatase (Sigma) diluted 1/1000 was added to each strip, incubated for 1 h at room temperature and the strips washed as before in TBS-T. Nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Roche) diluted 1/50 in alkaline phosphate buffer, pH 9.5, were used to detect bound antibody.

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