Small ruminant lentivirus proviral sequences from wild ibexes in contact with domestic goats

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Small ruminant lentiviruses (SRLVs) are widespread amongst domesticated goats and sheep worldwide, but have not been clearly identified in wild small ruminants, where they might constitute an animal health risk through contamination from local domesticates. SRLV proviruses from three ibexes from the French Alps are described and sequences from their gag gene and long terminal repeats (LTRs) were compared with sequences from local goats and goat/ibex hybrids. The ibex and hybrid proviruses formed a closely related group with <2% nucleotide difference. Their LTRs were clearly distinct from those of local goats or reference SRLV sequences; however, their gag sequences resembled those from one local goat and reference sequences from caprine arthritis encephalitis virus rather than visna/maedi virus. One SRLV-positive ibex from a distant site shared similarities with the other ibexes studied in both its gag and LTR sequences, suggesting that a distinct SRLV population could circulate in some wild ibex populations.

Small ruminant lentiviruses (SRLVs) constitute a varied group of retroviruses, including caprine arthritis encephalitis virus (CAEV) and visna/maedi virus (VMV), that are widely spread among domestic goats and sheep throughout the world with few exceptions, mostly in island locations (Pepin et al., 1998; Peterhans et al., 2004). SRLV origins remain obscure (Katzourakis et al., 2007; Querat et al., 1990) and similar viruses have not been found in wild small ruminants despite the frequent capacity of lentiviruses to cross species barriers, perhaps related to their high within-species genetic variation and propensity for recombination (VandeWoude & Apetrei, 2006). SRLVs are usually transmitted from dam to offspring by infected monocytes in milk or colostrum, but contact or venereal transmission are also possible, as in the classical epizootic of VMV in Iceland following the introduction of contagious Karakul rams (Palsson, 1972); contagion between goats is greatly reduced if barriers prevent physical contact (Peterhans et al., 2004). The possibility of transmission of SRLV to endangered wild small ruminants at a site in the French Alps where a small herd of heavily infected goats share grazing grounds with a small group of wild ibexes (Capra...
ibex) was investigated. Close contact between the goats and ibexes studied here has been confirmed by routine and specific observations (Richomme et al., 2006) and the birth of several goat/ibex hybrids. Proviral
after 1 year (age approximately 20 months) provided DNA
separate but adjacent pen. Peripheral blood samples taken
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CAEV on arrival and were housed together; the fourth was,
on 17 April 2002). Three animals were seropositive for
amplified using
A 512 bp canonical fragment of the
gene comprising
was described previously (Guiguen et al., 2000) and onto
EDTA for previously
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The small group of ibexes, initially derived from a single
pregnant female and isolated from other ibex groups, has
been observed by the French wildlife survey over 12 years
on high pastures shared with the domestic goat flock. The
first offspring was a male (ibex
1) that remained
in the group until his death when his son (ibex
3) replaced him. Animals in the group (up to seven in
total) were trapped at intervals for veterinary and serological surveillance. The founder female died shortly
before our study. The domestic goat flock had a high
turnover, as animals were frequently culled and replaced,
and typically consisted of five to seven adult nannies and
their kids. They were in regular summer contact with the
ibexes and hybrid offspring often resulted. At the time of
sampling, five of six adult nannies were seropositive for
CAEV. Four hybrid females born to different dams in the
same year at an age of approximately 8 months were
purchased before they had been released to their summer
pastures where they could contact the ibex population.
They were maintained in stables at the Lyon Veterinary
School for 2 years (authorization for animal testing no.
5759; experimental protocol approved by the Ethics
Committee of the Ecole Nationale Vétérinaire de Lyon,
on 17 April 2002). Three animals were seropositive for
CAEV on arrival and were housed together; the fourth was,
and remained, seronegative for CAEV and was kept in a
separate but adjacent pen. Peripheral blood samples taken
after 1 year (age approximately 20 months) provided DNA
for investigation of SRLV proviral sequences. A third ibex
(ibex
2), which was positive for SRLV by PCR, was
sampled at a site >100 km away.

Peripheral blood mononuclear cells (PBMCs) were
centrated from 10–50 ml blood by Ficoll centrifugation
(Narayan et al., 1983) and, when viable, monitored for the
development of syncytia indicative of SRLV infection
(Narayan et al., 1980) in simple culture or co-culture with
susceptible goat synovial membrane cells. Genomic DNA
was extracted from 5 × 10^6 uncultured PBMCs using the
DNeasy blood and tissue kit (Qiagen) according to the
manufacturer’s instructions. DNA concentration and
quality were determined spectrophotometrically and stocks
were kept at −70 °C before amplification of proviral

A 512 bp canonical fragment of the gag gene comprising
the MA region and the first 24 nt of the capsid region was
amplified using Pfu polymerase (Promega) by nested PCR

with primers and conditions described previously
(Chebloune et al., 1996). Primers, numbered according to
the CAEV reference strain Co (CAEV-Co) (Saltarelli et al.,
1990), were: GEX5 (5′-GAAGTGTGCTGGAGAGGT-
CTTG, nt 393–416) and GEX3 (5′-TGCCCCATCCAT-
GTTAGCTTGTGC-3′, nt 1291–1268) for the first
amplification; and GIN5 (5′-GATAGACATGGCAG-
GCAAGT-3′, nt 524–546) and GIN3 (5′-GAGGCCATGC-
TGCTTACGTCTG-3′, nt 1036–1013) for the second
amplification.

Proviral LTRs were amplified by single-round PCR using
primers U35 (5′-GTTAGACATGGCAGAGGAC-
3′, nt 889–8843) and U53 (5′-GCTGCGAGACCTGCT-
TGGTATTGTC-3′, nt 163–139).

The PCR mix in a final volume of 50 μl consisted of 1×
Pfu DNA polymerase buffer [20 mM Tris/HC1 (pH 8.8),
10 mM KCl, 10 mM (NH4)2SO4, 2 mM MgSO4, 0.1 mg
nuclease-free BSA ml−1], 200 μM dNTP, 1.25 U Pfu DNA
polymerase, 0.5 μM each primer and 500 ng DNA. Each
amplification included a positive control (CAEV-Co-
infected GSM cells) and negative control (ultrapure water)
run in parallel. All PCR products were observed by
electrophoresis through 1 % agarose gel containing 1 μg
ethidium bromide ml−1 in 1×TAE buffer.

Amplicons from three independent PCRs from each animal
were purified using the Montage PCR kit (Millipore), then
A-tailed by incubation for 10 min at 72 °C with Taq
polymerase and dATP for cloning into the pGEM-T Easy
Vector System 1 (Promega). Ligation products were used
to transform MAX Efficiency DH5α chemically competent
cells (Invitrogen) and plasmid DNA was extracted using
the Plasmid Mini kit (Qiagen) and observed by EcoRI
digestion to identify inserts with the expected size. With
the exception of gag sequences from hybrid
3, where only
two clones could be obtained, three to seven clones per
locus were sequenced for each animal using an ABI Prism
3100 Genetic Analyzer at the ‘Sequencing Technical
Platform’, IFR128, Lyon, France, using GIN5′ and GIN3′
primers for the gag gene and U35 and U53 primers for the
LTR region. GenBank accession numbers are given in the
legend of Fig. 1.

The individual sequences from the different animals were
aligned with reference sequences by using CLUSTAL W
(Thompson et al., 1994), with manual adjustment where
necessary. Different sequences from individual animals
generally varied by only 0.2–1.4 % (Table 1). Goat
3 provides an interesting exception, with a single clone of gag
(clone #4) that diverged by 8 % from the mean of the otherive clones, leading to an intra-animal divergence of 3.5 %.
In contrast, the LTR of goat #1 was virtually identical in all
three clones. The ibex and hybrid proviral sequences varied
between different animals by <2 %, whereas those from
goats formed a diverse group with >7.5 % variation
between animals. Finally, ibex and hybrid proviruses were
similar in their gag and LTR sequences (<1.9 % variation)
and both differed more considerably from the goat
sequences in both gag (>6.5%) and LTR (approx. 4%) sequences; see Table 1. The nature of these variations is shown in phylogenetic trees (Fig. 1) constructed using the neighbour-joining method (Saitou & Nei, 1987), with bootstrap values determined over 1000 iterations (Felsenstein, 2002). Trees were formatted using the program NJPLOT (Perriere & Gouy, 1996). The gag sequences (Fig. 1a) show that proviral sequences from goats #2 and #3 clustered together, with the sequences of clone #4 from goat #3 and CAEV-Co being more distantly related. All sequences from ibexes and hybrids bunched together with those from goat #1 on a separate branch, well supported by a bootstrap value of 998. This might suggest a common origin for these proviruses, but the LTR sequences (Fig. 1b) of goat #1 clustered with those from goat #2 and CAEV-Co, whereas the ibex and hybrid proviruses formed a distinct group, strongly supported by the bootstrap values. Trees constructed using different algorithms produced very similar results (not shown) and homologous sequences from reference VMV fell far outside either grouping (not shown).

The gag proviral sequences from ibexes, hybrids and goat #1 showed a 6 nt deletion compared with CAEV-Co and the other goats in this study, leading to a 2 aa deletion in the MA protein (Fig. 2). This deletion is not of itself responsible for the division into two groups on the tree, because when the deletion was artificially replaced in the sequences, the resulting trees were not significantly altered (not shown). Surprisingly, this marker was present in all the hybrids sequenced, despite their having different mothers, and in all the ibex sequences, including those originating from a female (ibex #2) from a distant site. Analysis of synonymous and non-synonymous nucleotide changes using the Datamonkey

![Fig. 1. Neighbour-joining trees of an approximately 0.5 kb fragment of the gag region (a) and 0.3 kb fragment of the LTR region (b) of proviruses from the studied animals and CAEV-Co. Horizontal lengths are proportional to the estimated genetic distance between the sequences; bar, 0.5% divergence. Bootstrap values derived from 1000 replicates are shown on the trees. Sequences are given as: G, goat; H, hybrid; I, ibex, followed by animal number and clone number.](image-url)
The marker deletion in gag MA was shared by one domestic goat and, even when the deletion was artificially restored in the sequences, the gag from goat #1 differed from those from hybrids. It was found that the ibexes and the hybrids carry a subset of proviral sequences that distinguish them from most sequences from domesticated animals. The 5′ region of the gag gene was studied in this report. This region is known to be both genetically and antigenically variable (Grego et al., 2005; Pisoni et al., 2006), but has unfortunately not been exploited for taxonomic studies of SRLV that use the CA region of the gag gene and/or pol sequences (Leroux et al., 1995, 1996; Shah et al., 2004a; Zanoni, 1998). All sequences from ibexes and hybrids shared a striking marker deletion of 6 nt corresponding to the absence of a glutamine–glutamic acid pair, which has not been described in CAEV type sequences, although it has been documented in one otherwise quite distinct VMV-type sequence from a Spanish sheep (Reina et al., 2005, 2006). The sequences were more closely related to typical published CAEV sequences than to VMV sequences (not shown), but a more precise taxonomic situation of our sequences in the SRLV group is not yet possible.

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### Table 1. Origins and characteristics of the gag and LTR sequences from the studied animals

<table>
<thead>
<tr>
<th>Animal (age/sex)*</th>
<th>CAEV serology</th>
<th>Sequence characteristics</th>
<th>LTR</th>
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<tr>
<td></td>
<td></td>
<td>gag</td>
<td>LTR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No. clones</td>
<td>Intra-animal divergence†</td>
</tr>
<tr>
<td>Goat</td>
<td></td>
<td>1 (ND/F)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 (ND/F)</td>
<td>4</td>
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<tr>
<td></td>
<td></td>
<td>3 (ND/F)</td>
<td>6</td>
</tr>
<tr>
<td>Goat/ibex difference:§</td>
<td></td>
<td>1 (12 y/M)</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 (6 y/F)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 (7 y/M)</td>
<td>4</td>
</tr>
<tr>
<td>Ibex/ibex difference:§</td>
<td></td>
<td>1 (20 mo/F)</td>
<td>4</td>
</tr>
<tr>
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<td>3 (20 mo/F)</td>
<td>2</td>
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<tr>
<td>Goat/ibex difference:§</td>
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<td>5</td>
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<td>1 differed from 2</td>
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<tr>
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<td>1 differed from 2</td>
<td>1</td>
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</table>

*y, Years; mo, months; M, male; F, female.
†Percentage nucleotide divergence between different clones from the same animal (mean ± SD).
‡Percentage nucleotide divergence between animals of the same type (goat, ibex, hybrid).
§Intergroup (goat/ibex/hybrid) nucleotide divergences are indicated in bold.
ND, Not determined.
Fig. 2. Alignment of deduced amino acid sequences of the gag region of proviruses from the nine studied animals with the sequence of clone #2 of goat #2 (Goat2-2) used as a reference of the studied population. Only amino acids differing from the Goat2-2 sequence are shown. Dots indicate identity with Goat2-2 and dashes indicate deletions.
those of the ibexes and hybrids and resembled those from other domestic goats (Fig. 1b). The hybrids must have acquired their infection from their dams or by horizontal transmission within the small flock, as they had not encountered wild ibexes at the time of purchase. The ibex-type LTR has never been reported from goats or sheep, but must have been present either in a culled animal or at low frequency within the flock. It is, however, the major sequence in wild goats and the hybrids. This might represent selection of a variant sequence in a different host context; however, passage of CAEV and VMV between sheep and goats is not accompanied by major genetic changes in the virus (Pisoni et al., 2005; Shah et al., 2004a, b), although sheep and goats are further apart genetically than goats and ibexes. Alternatively, a variant of SRLV may be present in some wild ibexes, as suggested by the very similar, though distinct, sequences from ibex #2 from a distant site. The rarity of ibexes that are seropositive for SRLV suggests that any such virus must be poorly cross-reactive with CAEV antigens used for testing; indeed, two of our ibexes carrying provirus detectable by PCR were seronegative by ELISA. The infectious virus isolated from samples from ibex #1 may prove to be a source of specific ibex SRLV sequences for more accurate testing of wild populations.

Acknowledgements

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