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Generation of a molecular clone of an attenuated lentivirus, a first step in understanding cytopathogenicity and virulence

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ABSTRACT

Small ruminant lentiviruses infect goats and sheep, inducing clinical disease in a minority of infected animals. Following an eradication campaign, clinical cases may disappear in a population. The complete elimination of these lentiviruses is however difficult to achieve and the spreading of less virulent strains often parallels the elimination of their virulent counterparts. Here, we characterized three such strains isolated from a flock in the post-eradication phase. We completely sequenced their genomes, showing that one of the isolates was most probably the product of a recombination event between the other two viruses. By comparing the sequences of these isolates with those of virulent strains, we found evidence that particular LTR mutations may explain their attenuated phenotype. Finally, we constructed an infectious molecular clone representative of these viruses, analyzing its replication characteristics in different target cells. This clone will permit us to explore the molecular correlates of cytopathogenicity and virulence.

Introduction

Small ruminant lentiviruses (SRLV) are a complex phylogenetic group of retroviruses, comprising of the circulating strains of caprine arthritis encephalitis virus (CAEV) and Visna/maedi virus (VMV), which induce persistent infections in goats and sheep. Infected animals may develop pathological manifestations such as arthritis, pneumonia, encephalitis and mastitis. The incidence and severity depend on two principal factors, the genetic background of the infected animals and the virulence of the infecting SRLV strains. The genetic background of the animals appears to influence the susceptibility to infection and has an important impact on the frequency and severity of the induced lesions (Heaton et al., 2012; Larruskain and Jugo, 2013; White et al., 2014; Ruff and Lazary, 1988; Dolf and Ruff, 1994). The virulence of SRLV is highly variable, encompassing few, highly virulent strains and a majority of strains with low or no apparent virulence (Gudmundsson et al., 2005; Oskarsson et al., 2007; Reina et al., 2009; Angelopoulou et al., 2008, 2006; Barros et al., 2004; Glaria et al., 2012).

The principal target cells are monocytes and macrophages (Narayan et al., 1982). Furthermore, dendritic cells are important target cells *in vivo* (Ryan et al., 2000). In monocytes, virus replication is restricted and productive replication starts after the maturation of monocytes to macrophages (Narayan et al., 1983).

SRLV are phylogenetically divided into 5 groups A–E, which comprise different subtypes, based on their *gag* and *pol* sequences (Shah et al., 2004a). The majority of these subtypes are able to cross the species barrier between goats and sheep, and vice-versa, under field conditions (Shah et al., 2004b; Pisoni et al., 2005; Bertoni and Blacklaws, 2010).

In Switzerland, an eradication campaign began in the 1980s which focused exclusively on goats. This campaign became mandatory in 1998, and resulted in a drastic reduction in seroprevalence (from 60–80% to less than 1%). Simultaneously, the clinical cases of arthritis completely disappeared.

Nowadays, in spite of the success of this campaign, seroconversions still occur on regularly and SRLV has been isolated from different goats and sheep flocks (Cardinaux et al., 2013; Deubelbeiss et al., 2014). The main circulating viruses belong to

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the subtypes A4 and A3, and appear to be of low virulence, with the exception of the mammary gland, where histopathological lesions were detected in adult infected goats (Deubelbeiss et al., 2014).

In this work, we focused on three particular SRLV A4 field isolates, co-circulating in a previously described flock of goats and sheep (Cardinaux et al., 2013). We aimed to sequence the complete genomes of these viruses, permitting us to characterize these particular isolates and to construct a replication competent molecular clone.

Results

Sequencing

In a previous study, we described the phylogenetic characteristics and *in vitro* replication properties of 3 SRLV isolates, obtained from the milk cells of one goat and the PBMC of two sheep living in a mixed flock. In this work, we aimed to sequence the complete genome of the three isolates. Since the 5′ and 3′ ends of the genomes were unknown, we first used two primers (Table 1, primer LTR-F and LTR-R) located in the *env* region and in the LTR, to amplify and sequence the U3 part of the 3′ LTR. Both LTR being identical, we could then determine the 5′ end of the genome.

The complete consensus sequences of g6221, s7385 and s7631 were determined by amplifying and sequencing five overlapping PCR products, using different primers (Table 1).

The three isolates have a total length of 9464 bp; all open reading frames (ORF) corresponding to the main and accessory genes were present and intact (GenBank accession numbers: KT453988 for g6221, KT453989 for s7385 and KT453990 for s7631). Additionally, the derived amino acid sequence of the dUTPase encoding region showed an intact active site compared to the phylogenetically distant equine infectious anemia virus (EIAV) or feline immunodeficiency (FIV) dUTPase proteins (Fig. 1s Supplementary material). The dUTPase inactivating E to G mutation described by Turelli et al. (1996) at position 109 in the CAEV-CO strain was intact (E) in our molecular clone.

Phylogenetic analysis and comparison between the 3 isolates

The 3 isolates could be classified as SRLV subtype A4, based on phylogenetic analyses performed with the entire sequences (Fig. 2) or the *gag*, *pol* and *env* genes, as well as the LTR (data not shown).

A pairwise DNA distance analysis over the complete genome (SimPlot; Lole et al., 1999), shown in Fig. 1, confirmed the phylogenetic analysis and revealed that the g6221 was much closely related to a previously sequenced SRLV-A4 isolate (GenBank: AY445885) than to the prototypic sequences CAEV-CO (SRLV-B1 genotype, GenBank: NC_001463.1) and VMV 1514 (SRLV-A1 genotype, GenBank: M60610.1). As expected, the env gene showed the highest divergence between the g6221 virus and the reference strains, along with the LTR sequence, which was quite distant to its counterparts in the prototypic strains (Fig. 1).

Recombination event

Using the RDA-4 software, we demonstrated that isolate s7385 was most probably the product of a recombination event between the two parental viruses g6221 and s7631 (Martin et al., 2015). As shown in Fig. 3, the recombination event was located in the highly variable region encompassing the V4-V5 domains of env, between positions 7753 and 8311 of the alignment. The probability figures, using different methods, were 3.156×10^{-03} for RDP, 3.083×10^{-04} for MaxChi and 2.233×10^{-03} for Chimaera.

LTR analysis

The LTR sequences of the three viruses were highly similar to each other but, as shown in Fig. 1, quite distant to the prototypic SRLV-A and -B sequences. Different transcription factor binding sites were mapped in the long terminal repeat, such as 5 AP-1 sites, one AP-4, 2 AML(vis) and an E-box (Fig. 4).

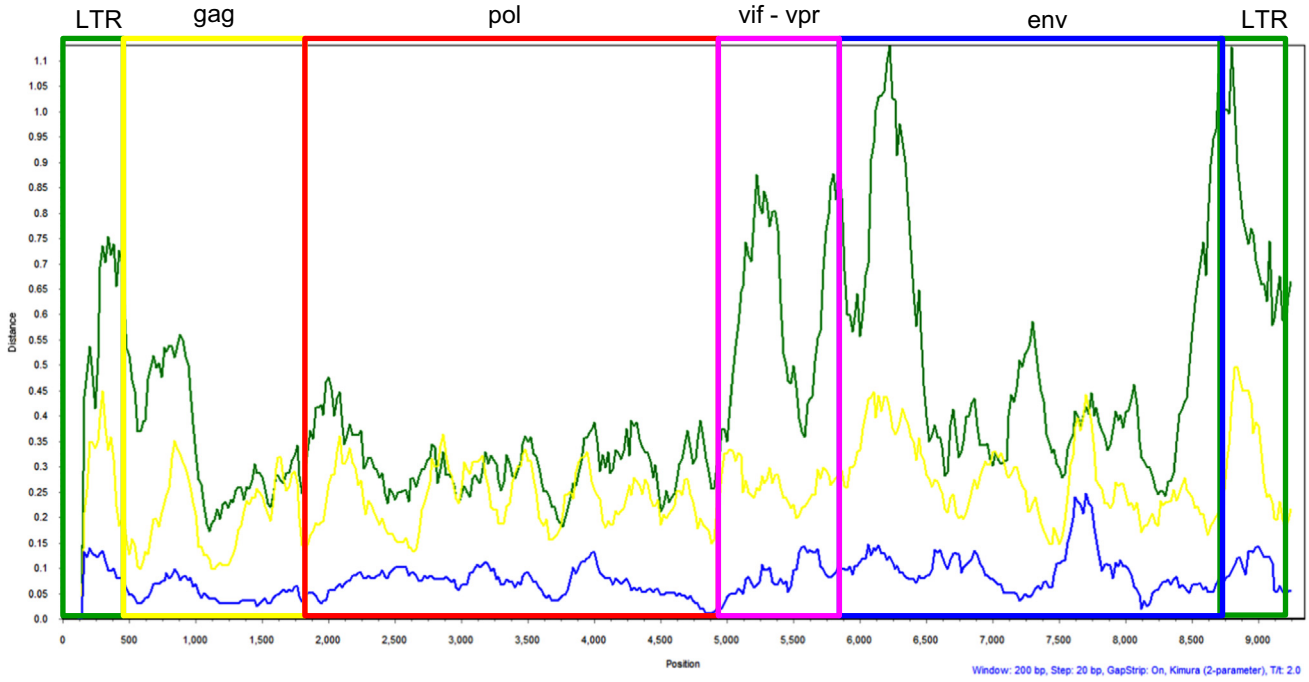

Fig. 1. A Pairwise DNA Distance analysis of the complete genomes (SimPlot) was performed using the SimPlot software. Strains AY445885 (blue line), CAEV-CO (green line) and MVV 1514 (yellow line) were compared to the SRLV-A4 isolate g6221. The two long terminal repeats (LTR) and the genes gag, pol, vif, vpr (former tat) and env are shown on top.

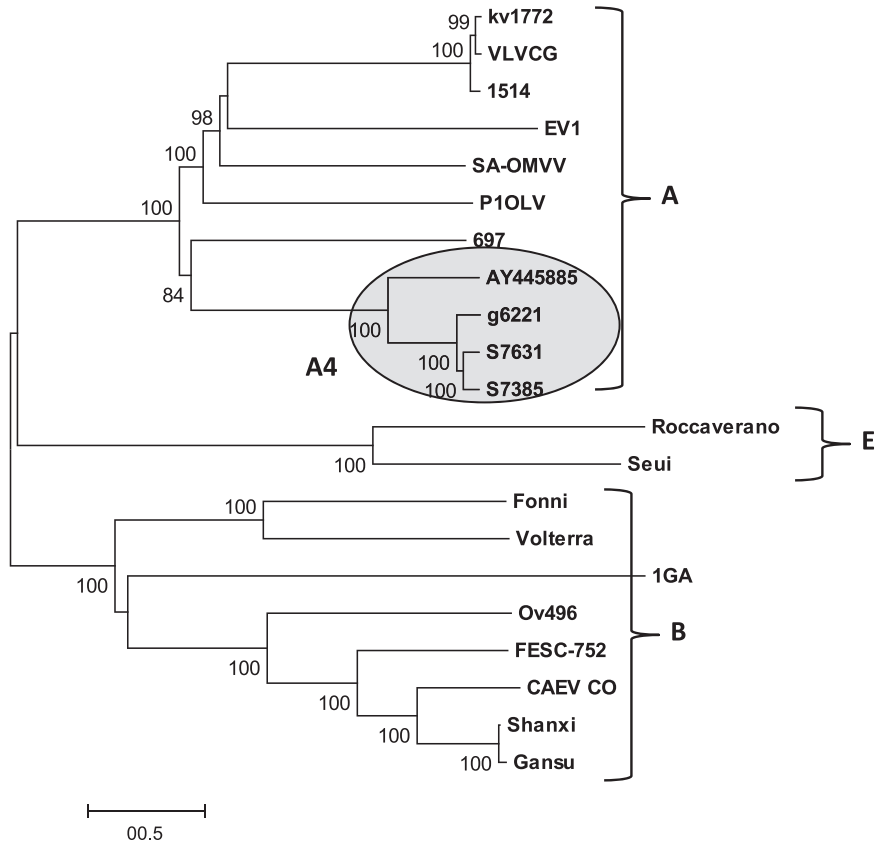

Fig. 2. Phylogenetic analysis of SRLV complete genomes. The genome of the molecular clone g6221 and the two sheep isolates s7631 and s7385 are clearly distinct but closely related to the previously sequenced SRLV-A4 Swiss isolate AY445885.

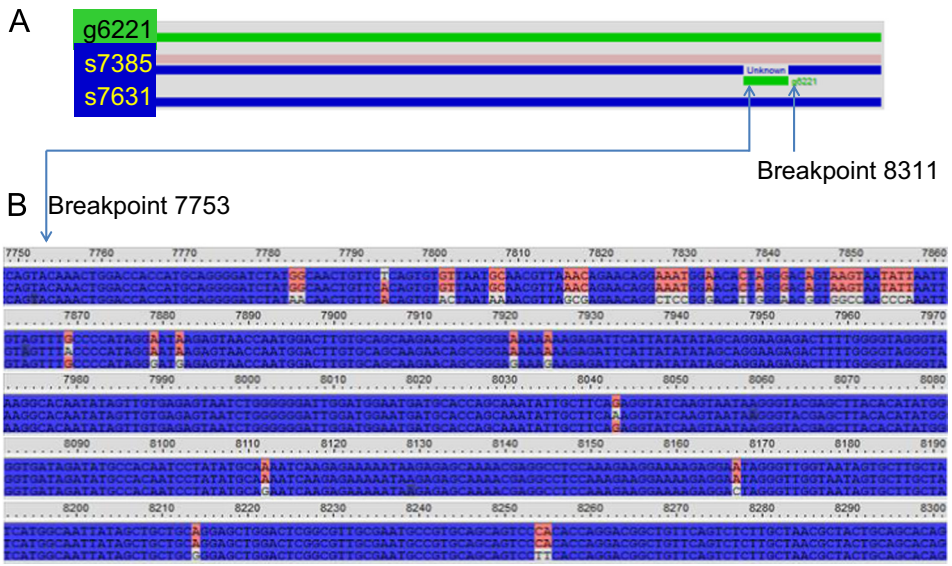

Fig. 3. (A) schematic representation of the viral genomes g6221 (green line) and s7385 and s7631 (blue lines). The green bar underneath the s7385 genome highlights the recombined fragments, most probably originated from the env gene of the g6221 genome. (B) The sequence alignment of the three genomes, in the region encompassing this recombination event illustrates the striking similarity between the g6221 and s7385 genome. This region encompasses the V4-V5 variable regions of env. Identical bases between the goat isolate g6221 and the sheep isolate s7385 are highlighted in red. The RDA-4 software identified the breakpoints at positions 7753 and 8311.

By comparing these elements with the consensus sequences defined in the literature, we noticed the presence of several discordances. Indeed, only a unique AP-4 site and one AML (vis)-b site contained the perfectly conserved core consensus sequences CAGCTG and TAACCGCA, respectively. In contrast, the AML(vis)-a site was disrupted in all three isolates by the insertion of a thymidine residue (underlined) between the two adjacent adenines (TA<u>T</u>ACCGCA). In addition, the duplication of this AML (vis) site present in some SRLV strains, such as the Icelandic isolates, was not observed in our isolates.

All the AP-1 sites contained at least 1 mutation compared to the defined consensus sequence TGA(C/G)TCA. The AP-1c site contained a T to A mutation at position 5 but, in contrast to the other AP-1 sites, this particular sequence was highly conserved

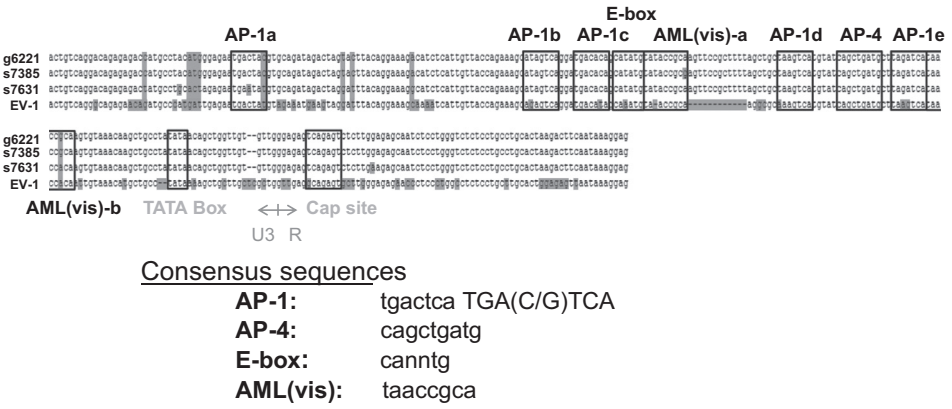

Fig. 4. Alignment of LTR sequences. The border between the U3 and R regions are indicated () as well as the TATA box and the cap site (light gray). (a) LTR sequences of strains g6221, s7385 and s7631 were compared to the reference strain EV-1. Potential transcription binding sites described in the literature are boxed. Mutations affecting the consensus sequences and potentially disrupting the promoter activity of the LTR of these particular isolates are shaded in gray. (b) Consensus sequences of the mapped transcription factor binding sites.

between SRLV strains, suggesting a potential functional role. Strikingly, the sequence of an E-box, described as one of the most conserved LTR elements in the SRLV of caprine and ovine origin, was mutated in these three isolates.

Generation of a synthetic, replication competent molecular clone of g6221

Sequence analysis and the subsequent allocation of putative functional genomic regions enabled us to draw interesting working hypotheses centered on a series of mutations in the LTR, which may explain the attenuated phenotype. Further elucidation of these viruses is made possible by the generation of an infectious molecular clone, an indispensable tool allowing the dissection of mechanisms of cytopathogenicity and virulence. To understand the molecular properties of SRLV A4 viruses still circulating in goats in the post-CAEV eradication campaign phase, we selected the g6221 isolate to generate a replicating molecular clone.

The complete virus genome, flanked by two unique restriction sites NotI and XhoI, was chemically synthesized and cloned in a modified pBR322 plasmid. Transfected primary GSM cells provided the virus stock used in the studies described below.

Replication characteristics of the molecular clone and the original field isolate in cell culture

We compared the molecular clone pBR322-g6221 with the original field strain *in vitro* by infecting primary goat macrophages and GSM cells.

To compare the replication characteristics of the two preparations, we monitored the RT activity in the supernatant of the infected cells at regular intervals and up to 15 days post-infection.

As shown in Fig. 5A, the kinetics of RT activity accumulation in the supernatant of infected GSM cells was similar for both viruses and a repeated measures analysis of variance (ANOVA) did not reveal a significant difference between the two virus preparations. In contrast, in goat macrophages, the molecular clone showed a significant, delayed increase of RT activity ($p \leq 0.0152$, ANOVA), reaching, however, comparable RT activity levels by the end of the experiment Fig. 5B.

The microscopic examination of infected GSM cells revealed the presence of mild cytopathic effects with both viruses, characterized by the presence of small syncytia at 15 days post-infection (data not shown). In contrast to the mild effects observed in infected GSM cells, both virus preparations showed

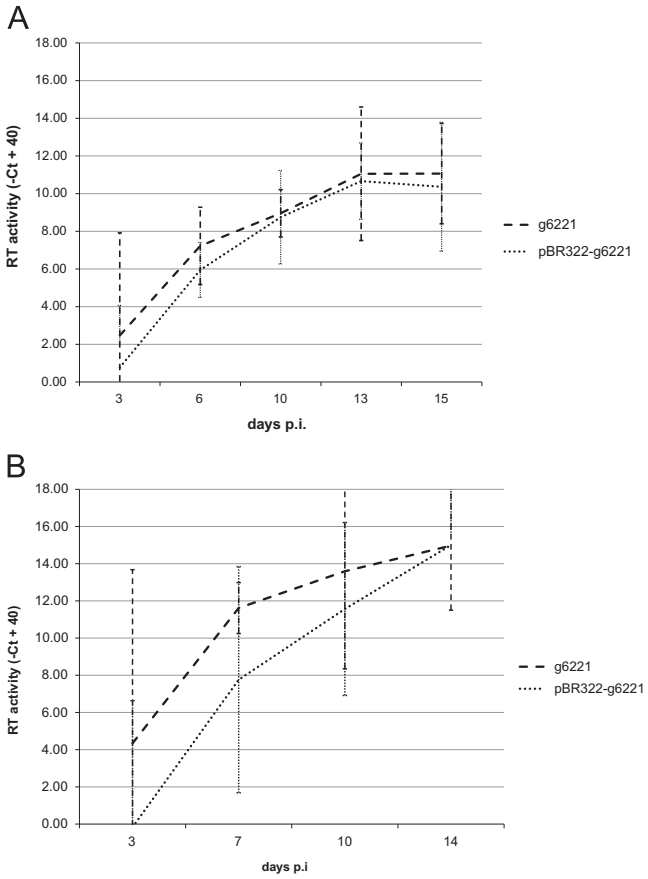

Fig. 5. (A) Replication characteristics of the molecular clone pBR322-g6221 compared to the original field strain g6221. Following infection of GSM cells, the RT activity in the cell culture supernatant was monitored by PERT assay. Error bars represent the 95% confidence intervals as determined with the Confidence-T function. (B) Replication characteristics of the molecular clone pBR322-g6221 compared to the original field strain g6221. After infection of primary goat macrophages, the RT activity in the cell culture supernatant was monitored by PERT assay. Error bars represent the 95% confidence intervals as determined with the Confidence-T function.

marked cytopathic effects in primary goat macrophages. In fact, the infected cultures showed several pyknotic cells and a marked decrease in the number of morphologically intact cells, as already described in previous studies (Cardinaux et al., 2013; Deubelbeiss et al., 2014).

Electron microscopy analysis

Once confirmed that the generated molecular clone behaved similarly to the original field isolate, we proceeded with the analysis of the ultrastructural changes induced by the g6221 molecular clone in infected cells. We infected GSM and primary goat macrophages with this virus, which is supposed to be mildly cytopathogenic at least in GSM cells; as a control, we infected the same cells with the highly cytopathogenic CAEV-CO molecular clone, a prototypic virulent SRLV group B virus. The results obtained in macrophages did not reveal any difference between these two viruses. In both preparations, the number of macrophages was reduced compared to the controls, and it was difficult to find sufficiently preserved cells for a morphological analysis. Few viral particles appeared in the cytoplasm of the remaining, strongly vacuolated macrophages or in the extracellular space near these cells. Contrastingly, the results obtained with infected GSM cells revealed a striking difference between these two viruses. The integrity of the cells was not affected by the viruses and numerous intracellular viral particles could be observed in both preparations. The viral particles detected in GSM cells infected with the highly cytopathogenic CAEV-CO had a circular shape, defined by the accumulation of electron dense material, as described for immature lentiviral particles. A virus envelope was barely visible and the immature particles appeared to arrange themselves in circles, distributed around intracellular membranes (Fig. 6A). Budding of mature virus particles was noted at the cell surface. In contrast, GSM cells infected with the SRLV-A4 virus g6221 showed a completely different picture. The numerous intracellular viral particles detected were mostly mature, displaying a distinct, torus-shaped capsids surrounded by a clearly visible viral membrane. The majority of these mature viral particles were concentrated in intracytoplasmic vesicles surrounded by a membrane and containing a large number of enveloped mature viral particles (Fig. 6B).

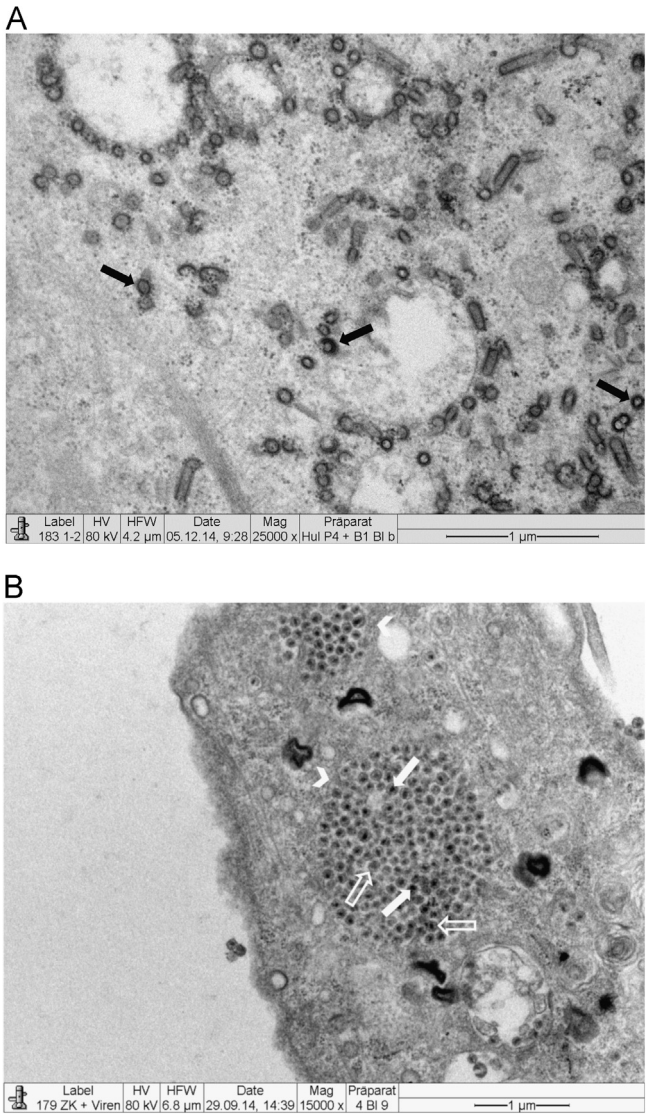

Fig. 6. (A) Electron microscopy analysis of GSM cells infected with the CAEV-CO molecular clone. Cells were analyzed at 9 days p.i. and showed several immature viral particles characterized by a typical accumulation of electron dense material (black arrows) and without a noticeable viral envelope. (B) Electron microscopy analysis of GSM cells infected with the molecular clone pBR322-g6221. The cells were analyzed at 9 days p.i. and showed numerous mature viral particles with a distinctive torus-shaped capsids (white arrows) surrounded by a clearly visible viral membrane (white empty arrows). Several intracytoplasmic vesicles surrounded by a membrane and containing a large number of viral particles were present (white arrowheads).

Discussion

The Swiss CAEV eradication campaign was highly successful in eliminating clinical cases of arthritis in the goat population, as well as in reducing the seroprevalence of SRLV genotype B infected goats well below the 1% mark (Bertoni, 2007). This notwithstanding, SRLV subtype A4 and A3 are still circulating in Swiss goats and sheep, potentially undermining the success of the eradication campaign. The absence of clinical cases of arthritis in goats in the last 20 years strongly argues in favor of a drastic attenuation of these viruses. However, recent observations indicate that these viruses may have a preferential tropism for the mammary gland, where they were shown to induce histopathological lesions (Deubelbeiss et al., 2014). We therefore decided to characterize these particular viruses at the molecular level, preparing the tools that will permit us to explore the molecular correlates of attenuation, the particular tropism of these viruses for the mammary gland, and to estimate the potential danger of reversion to full virulence.

To this end, we fully sequenced the 3 previously described SRLV A4 isolates g6221, s7631 and s7385 (Cardinaux et al., 2013). At first, the sequence analysis was unspectacular, showing that the three sequences were highly similar to each other and closely related to a previously reported SRLV A4 sequence (AY445885) (Shah et al., 2004). A more detailed analysis of their genomes revealed that the isolate s7385 was most probably the product of a recombination event between the two other viruses, centered on the V4-V5 region of *env*. This explains a previously noticed incongruence between the phylogenetic classification of these 3 viruses in the pol and env regions. Indeed, the s7631 and s7385 pol sequences clustered together and were significantly distinct from the pol sequence of g6221, whereas the phylogenetic analysis of the env region indicated that the g6221 and s7385 were closely related but divergent from the s7631 env sequence (Cardinaux et al., 2013). The recombination event may have occurred in sheep #7631 previously shown to be doubly infected (Cardinaux et al., 2013). Recombination events occur quite frequently in individuals multiply infected with genetically distinct lentiviruses, and this has profoundly influenced the epidemiology of, for instance, HIV, where unique and circulating recombinant forms are widely spread (Taylor et al., 2008). Recombination events between SRLV were first described in experimentally infected animals, pointing to the env gene as a privileged recombination site (Andresdottir, 2003). Further reports showed that recombinant viruses could also be detected in naturally infected animals; in these reports, the env

gene was also frequently targeted by these recombination events (Ramirez et al., 2011; Pisoni et al., 2007; L'Homme et al., 2015).

We then analyzed the virus sequences for the presence of intact ORF, looking in particular for genes encoding well known virulence factors. The ORF for the vif and vpr (tat) genes were intact and the derived dUTPase amino acid sequence did not show known inactivating mutations (Turelli et al., 1997, 1996). This suggests that the attenuated phenotype of these viruses could not be attributed to deletions in these genes, as is the case for SRLV belonging to the E group or an attenuated molecular clone of the B1 subtype lacking an intact dUTPase ORF (Turelli et al., 1997; Reina et al., 2009). On the contrary, the LTR region of these three viruses and an additional Swiss SRLV A4 isolate (AY445885; Shah et al., 2004) presented several mutations in potentially important transcription factors binding sites. These mutations may well relate to the attenuated phenotype *in vivo*, characterized by the lack of arthritis in infected animals, contrasting with a surprisingly high viral load in the mammary gland. Similarly, these LTR mutations may explain the particular cell tropism and behavior of these viruses *in vitro*, characterized by a mild cytopathogenicity and a more vigorous replication in GSM than LSM cells and a similar replication efficiency and high cythopathogenicity in goats and sheep macrophages (Cardinaux et al., 2013; Deubelbeiss et al., 2014).

With the exception of a unique AP-4 site and the AML(vis)-b site, the consensus sequences of several transcription factor binding sites, previously described to control SRLV replication, were mutated in these A4 SRLV strains. The conserved AP-4 was shown to be very important, or even indispensable for the promoter activity of the LTR (Hess et al., 1989; Juganaru et al., 2010). The AP-1a consensus sequence, responsible for the TPA responsiveness of the LTR *in vitro* (Gabuzda et al., 1989), showed several mutations in the three Swiss sequences analyzed, one of which, the G to A transition in position 2, was shown to impair binding of transcription factors to the homologous sequence in the EV-1 LTR (Sutton et al., 1997). The AP-1c had one single mutation compared to the consensus sequence. The fact that this site was perfectly conserved in the LTR sequences of several SRLV isolates suggests a potential functional importance for this site. Together with the AP-1d site, AP-1c was postulated to influence the basal and TPA induced activity of the Visna virus in U937 cells (Gabuzda et al., 1989; Hess et al., 1989). The AP-1d of the Swiss isolates matched those of the Icelandic isolates. The AP-1e site showed 3 mutations compared to the AP-1 consensus sequence and, together with the AP-4 site, was shown to be the most important control element for the activation of the Visna virus LTR (Gabuzda et al., 1989; Hess et al., 1989). The adjacent AML(vis)-a consensus sequence was disrupted by the insertion of a T in position 3 in all Swiss isolates, suggesting a potential functional impairment of this site. This AML (vis) element is located in a region of perfect homology between VMV and CAEV strains (Hess et al., 1986, 1989) and was shown to bind transcription factors in the EV-1 strain (Sutton et al., 1997). Finally, the sequence of a perfectly conserved E-box (CAAAT), present in all analyzed VMV and CAEV strains, was mutated in the three Swiss strains. In spite of these mutations, however, their E-box still matched the consensus sequence CANNTG. This box, present in a duplicated sequence in the LTR of neurotropic VMV strains, was shown to determine the replication rates in sheep chorioid plexus cells (Oskarsson et al., 2007). Using the novel molecular clone, we will be able restore the E-box of the g6221 virus and test if the detected mutations are responsible for the impaired replication of the Swiss SRLV A4 isolates in *e.g.*, LSM cells (Cardinaux et al., 2013).

P1OLV, a highly attenuated Portuguese isolate, shows similar potentially attenuating mutations in the U3 region as the three Swiss strains mentioned above. Indeed, the AP-1a and AP-1b consensus sequences contain similar mutations as those observed in our isolates. In contrast, the AML(vis)-a consensus sequence and the adjacent CAAAT box are intact in this particular slow/low VMV strain. The P1OLV LTR was inserted in the LTR region of the neuropathogenic KV1772 clone, generating a chimeric virus with a dramatically impaired replication activity in SCP and lung cells compared to the parental KV1772 strain (Barros et al., 2005). The fact that the LTR of the Swiss isolates present even more mutations in functionally important LTR elements provides additional supporting evidence to the hypothesis that the LTR of these strains play a pivotal role in conferring an attenuated phenotype to these viruses. This is particularly important in view of the fact that, contrary to the primate lentiviruses, the LTR of SRLV have an inherently high basal activity, independent of Tat transactivation (Villet et al., 2003). On top of the series of mutations found in the U3 region, the LTR of the A4 Swiss isolates contained a 12 bp deletion in the R region of the LTR, previously described by Angelopoulou et al. (2008). Working with a flock which included both infected animals showing classical clinical signs and clinically healthy but infected sheep, the authors demonstrated that SRLV isolated from the clinically healthy animals had a characteristic 11 bp deletion in the R region not found in viruses isolated from the sheep showing clinical symptoms (Angelopoulou et al., 2006, 2008). A similar deletion in the R region is also present in the AF479638 sequence derived from the highly attenuated Portuguese isolate P1OLV (Barros et al., 2004). Interestingly, the impaired virulence of the Swiss SRLV A4 does not compromise their capacity to efficiently spread in an infected goat flock, to successfully cross the species barrier, to infect goats and sheep with similar efficiency, or to spread in geographically distant regions (Deubelbeiss et al., 2014; Cardinaux et al., 2013; Shah et al., 2004b, 2004a). This provides strong evidence that virulence and evolutionary success are distinct properties of SRLV. The construction of an infectious molecular clone of the g6221 SRLV A4 isolates is an important step that will permit us to test the hypothetical importance of the LTR in the replication characteristics and attenuation of these particular viruses. The molecular clone, based on the consensus sequence of g6221, replicated in GSM and LSM cells with the same efficiency as the original field isolate (Fig. 5A and data not shown), showing similarly mild cytopathic effects in these cells. It efficiently replicated in macrophages, inducing similar cytopathic effects in these cells as the original isolate. In contrast, the g6221 molecular clone showed a delayed replication kinetic in these cells, as demonstrated by the retarded appearance of RT activity in the supernatants. Considering the fact that the infectious dose was the same as in GSM cells, where the two viruses were undistinguishable, we must assume that the g6221 molecular clone does not represent the most efficient macrophagotropic virus sequence present in the quasispecies of the original field isolate.

To explore the ultrastructural changes induced by our mildly cytopathogenic SRLV A4 viruses and the highly cytopathic CAEV-CO molecular clone (a prototypic SRLV B1 virus), we infected GSM cells and macrophages with these viruses and examined the cells by electron microscopy. Both g6221 and CAEV-CO clones were too cytotoxic for macrophages to permit a morphological analysis of these cells. On the contrary, GSM cells infected with the g6221 molecular clone or its wild type counterpart were perfectly preserved and showed a striking difference in the virion's morphology and virus particle distribution compared to GSM cells infected with the SRLV B1 CAEV-CO (Figs. 6B and A, respectively). The images show that the vast majority of the SRLV A4 virus particles are mature and sequestered in particular subcellular regions referred to as virus containing compartments in HIV infected macrophages (Orenstein et al., 1988; Mariani et al., 2014). In contrast, the CAEV-CO viral particles were immature and

distributed in the cytoplasm around intracellular membranes. Differences in sequence and maturation kinetic of their Gag proteins may well explain these divergent morphologies. Indeed, Gag is considered the master protein coordinating the interactions of the forming virions with different intracellular membrane domains (Lingappa et al., 2014). Future work, based on this novel SRLV A4 clone, will allow the creation of chimeric Gag proteins, swapping different domains of the CAEV-CO and g6261 Gag proteins, therefore identifying the domains involved in this dramatically different phenotype. Similarly, swapping the Env proteins of these strains, will allow exploration and elucidation of the involvement of this protein in the described cytopathic effects.

Material and methods

Virus strains

The virus strain g6221 was isolated from a SRLV seropositive goat, and the strains s7385 and s7631 from two SRLV seropositive sheep, as previously described (Cardinaux et al., 2013). Briefly, milk macrophages or blood-derived macrophages were isolated and cultured, and the cell culture supernatant collected at regular intervals.

Proviral DNA was isolated from infected macrophages and sequenced. Briefly, goat or sheep macrophages were isolated from SRLV seronegative animals, as previously described (Cardinaux et al., 2013), and infected with strains g6221, s7385 and s7631. Six days post-infection, the cells were harvested, and DNA isolated as previously described (Cardinaux et al., 2013).

Whole genome sequencing (g6221, s7631, s7385)

The complete genomic sequences of strains g6221, s7385 and s7631 were obtained by amplifying 5 overlapping PCR products. The primers used are listed in Table 1. PCR fragments were generated using the LongRange PCR Kit (Qiagen AG, Hombrechtikon, Switzerland) in a 50 µl reaction volume and the following cycling conditions: activation at 93 °C for 3 min, 35–40 cycles of 15 s each at 93 °C, 30 s at annealing temperature and 1 min/kb at 68 °C, followed by 1 min at 68 °C. The PCR products were analyzed on a 1% TAE Agarose gel containing ethidium bromide.

If several bands appeared on the gel, the PCR products were separated on a 1% NuSieve GTG Agarose (Cambrex, Rockland, USA). The desired band was excised and purified with QIAquick Gel extraction Kit (Qiagen AG, Hombrechtikon, Switzerland).

The PCR products were cloned using the TOPO TA cloning kit (Life Technologies Europe B.V., Zug, Switzerland), and transformed into One Shot® TOP10 chemically competent cells (Life Technologies Europe B.V., Zug, Switzerland). The cells were grown overnight on LB plates supplemented with 50 µg/µl ampicillin (Sigma-Aldrich Chemie GmBH, Buchs, Switzerland). Several colonies were picked and cultured overnight in LB Medium supplemented with 50 µg/µl ampicillin.

The next day, plasmids were purified using the Quicklyse Miniprep Kit (Qiagen AG, Hombrechtikon, Switzerland), and partially sequenced by Microsynth AG (Balgach, Switzerland).

At least three plasmids per virus strain were later purified with the Plasmid Midi Kit (Qiagen AG, Hombrechtikon, Switzerland), and the inserts completely sequenced by Microsynth AG (Balgach, Switzerland). The obtained sequences were edited with the DNASTAR software (version 5), and analyzed with the clone manager software.

The 5 overlapping sequences were finally aligned using the clone manager software generating a consensus sequence.

Molecular clone pBR322-g6221

The complete g6221 genome was chemically synthesized and cloned (Eurofins Genomics, Ebersberg, Germany) in the pBR322 vector, which had been previously modified by inserting an oligo-linker encompassing the XhoI and NotI restriction sites.

The molecular clone was finally propagated in One Shot® TOP10 chemically competent cells (Life Technologies Europe B.V., Zug, Switzerland) and purified with Qiagen Plasmid Maxi Kit (Qiagen AG, Hombrechtikon, Switzerland).

Transfection and infection

The pBR322-g6221 plasmid was linearized with NotI (Fermentas GmbH, St. Leon-Rot, Germany), before transfection.

For transfection, primary goat synovial membrane cells (GSM) cells were seeded in 6-wells plates in Dulbecco's MEM (Biochrom AG, Berlin, Germany) supplemented with 0.5% FCS (GE Healthcare Europe GmbH, Glattbrugg, Switzerland), at a density of 2.5×10^5 cells per well. The cells were transfected with 400 ng plasmid DNA, using 6 ul Lipofectamine LTX-Plus Reagent (Life Technologies Europe B.V., Zug, Switzerland) per well.

After 24 h, the cell culture medium was replaced with Dulbecco's MEM supplemented with 10% FCS. One-week post-infection, the cell culture supernatant was collected and analyzed by real-time RT-PCR (Cardinaux et al., 2013).

Fresh GSM cells and goat macrophages (5×10^5 cells per well) were seeded in 6-wells plates and infected with pBR322-g6221 or the original g6221 field strain, using 1×10^7 viral copies per well. After 24 h, medium was replaced with Dulbecco's MEM

Table 1
Primers used to amplify the complete genome of the SRLV isolates g6221, s7385 and s7631.

Primer name	Sequence (5′- > 3′)	Annealing temperature (°C)	Position (relative to g6221)
s7385_s7631_g6221_1F	ACT GTC AGG ACA GAG AGA CCA TGC CTA CAT G	60	1
s7385_s7631_g6221_1R	ATC CAC TCC CGT AGA ATT TCT TGC ATT G		2858
s7385_s7631_g6221_2F	CAG CAA GCA TCC CAG GCT AAT ATG	52	1445
s7385_s7631_g6221_2R	ATG TCC ATA GGG CTT GTC		4991
s7385_g6221_3F	AGG GCC AAC CTT CTA TAC	53	3636
s7385_g6221_3R	ACC AGC ACC ATT CTT CAC		6895
S7631_3F	ATT TAG GAG TAG AAC ACA CAA C	50	4793
S7631_3R	ATT CTC TTA CCA CTT CTG CC		6274
s7385_s7631_g6221_4F	AGG GGG CTA GAG TAG TAA GAA ACT G	56	6089
s7385_s7631_g6221_4R	CTG CCT CTA ACA CTT GCT GC		8320
s7385_s7631_g6221_5F	GTA GAT GTG TAC AAA GAC CAG	58	7556
s7385_s7631_g6221_5R	CCT GCG AGA CCC GCT CCG GTG TTG C		9464
LTR-F	GTG ATA GAA GCA CCR GTG GA	60	8815
LTR-R	CCA CGT TGG GCG CCA GCT GCG AGA		365

supplemented with 10% FCS for GSM cells, or RPMI 1640 plus GlutaMAX™ (Life Technologies Europe B.V., Zug, Switzerland) with 5% goat serum (Sigma-Aldrich Chemie GmBH, Buchs, Switzerland) for macrophages.

The cell culture supernatant was collected at regular intervals and analyzed for RT activity by PERT assay, as previously described (Cardinaux et al., 2013). The cells were stained with Hemacolor® to visualize potential cytopathic effects (Merck, Darmstadt, Germany).

Sequence analysis

Phylogenetic trees comprising complete or partial genomic sequences of the SRLV isolates described in this manuscript and several representative SRLV sequences deposited in GenBank were constructed using the Neighbour-Joining (NJ) method implemented in MEGA with the Tamura-Nei gamma distance (Tamura et al., 2011). The statistical confidence of the topologies was assessed with 1000 bootstrap replicates (Felsenstein, 1993). Sequences were screened for putative recombination events using the RDP-4 software as described by Martin et al. (2015). Protein alignments were generated using Geneious version 8.1.7. (Kearse et al., 2012).

Transmission electron microscopy

Infected cells in culture were washed once with PBS prewarmed to 37 °C and subsequently fixed with 2.5% glutaraldehyde (Merck, Darmstadt, Germany) in 0.1 M cacodylate buffer (Merck, Hohenbrunn, Germany) pH 7.4 for 30 min at room temperature or overnight at 4 °C. After three washes in cacodylate buffer for 10 min each, cells were post-fixed with 1% OsO_4 (Chemie Brunschwig, Basel, Switzerland) in 0.1 M cacodylate buffer for 1 h at 4 °C and again washed three times with cacodylate buffer. Thereafter, cells were dehydrated in an ascending ethanol series and embedded in Epon, a mixture of Epoxy embedding medium, dodecenyl succinic anhydride (DDSA) and methyl nadic anhydride (MNA) (Sigma Aldrich, Buchs, Switzerland). Epon was polymerized for 5 days at 60 °C. Resin blocks were trimmed and regions of interest were selected based on semithin sections that were stained with 1% toluidine blue. Ultrathin sections of 90 nm were then obtained with diamond knives (Diatome, Biel, Switzerland) on a Reichert-Jung Ultracut E (Leica, Heerbrugg, Switzerland) and collected on collodion-coated 200-mesh copper grids (Electron Microscopy Sciences, Hatfield, PA, USA). Sections were double-stained with 0.5% uranyl acetate for 30 min at 40 °C (Sigma Aldrich, Steinheim, Germany) and 3% lead citrate for 10 min at 20 °C (Laurylab, Saint Fons, France) in an Ultrastain® (Leica, Vienna, Austria) and examined with a Philips CM12 transmission electron microscope (FEI, Eindhoven, The Netherlands). Micrographs were captured with a Mega View III camera using the iTEM software (version 5.2; Olympus Soft Imaging Solutions GmbH, Münster, Germany).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2015.09.027.

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