In Vitro Properties Of Small Ruminant Lentivirus Genotype E.

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Abstract
Small ruminant lentivirus genotype E lacks the dUTPase subunit and vpr-like gene. Two strains (Roccaverano and Seui) with identical genetic organization have been described, with the env HV1–HV2 domains being the most divergent. Although dUTPase and vpr-like deletions have been involved in the RT fidelity in non dividing cells, both strains were able to replicate efficiently in blood derived macrophages (BDM), while virus production of E1 subtype was reduced or abrogated in replicating fibroblastic-like cells. The transcriptional activity of genotype E was similar in these two cellular populations. When viral pseudotypes were generated with the env of both viruses, Roccaverano pseudotype displayed a paranuclear localization on BDM, suggesting a different mechanism of entry. Polymorphic GAS and TAS sites in the U3 region, further suggest that a population different from classically activated macrophages can be infected by these viruses, opening new insights into lentiviruses with low or null pathogenic potential.

Keywords:
Small ruminant lentivirus Genotype E dUTPase vpr-like Viral pseudotype Entry assay

Introduction

Caprine arthritis encephalitis virus (CAEV) and Maedi Visna virus (MVV) collectively known as small ruminant lentiviruses (SRLVs) are a genetically and antigenically heterogeneous group of lentiviruses that cause slow progressive infection in sheep and goats, which results in the development of a multisystemic disease involving lungs, brain, joints and mammary glands (Zink et al., 1987). SRLVs in vivo infect cells of the monocyte–macrophage lineage and do not infect T cells, in contrast to the related immunodeficiency viruses of human (HIV), monkeys (SIV), and cats (FIV) (Lyall et al., 2000). MVV and CAEV are transmitted from infected mothers to offspring by ingestion of infected colostrum and milk while transmission among adults occurs through direct contact (Peterhans et al., 2004). Although cross- species transmissions have been clearly demonstrated for many SRLV subtypes (Pisoni et al., 2005; Shah et al., 2004; Valas et al., 1997; Zanoni, 1998), it is widely accepted that the host adapted virus segregates in tissues that enable a more efficient transmission, as is well known the horizontal transmission of MVV in sheep through lung secretions (Pepin et al., 1998). Interestingly, at least for sheep, the typical gross and histopathological lesions of interstitial pneumonia can be observed as a result of MVV-like or CAEV-like isolates (Grego et al., 2002).

SRLVs can be classified into five phylogenetic groups: genotype A, comprising MVV-like strains (Shah et al., 2004), genotype B, including CAEV-like isolates (Pisoni et al., 2005), genotype C, which affects Norwegian small ruminants (Gjerset et al., 2007), genotype D, characterized at the pol gene level in Swiss and Spanish isolates (Reina et al., 2006; Shah et al., 2004) and genotype E, which includes Italian caprine isolates (Grego et al., 2007; Reina et al., 2009a). The latter group was first identified in asymptomatic goat herds in which the local Roccaverano breed was prevalent and the consequent prototype strain has been named Roccaverano. To date, genotype E comprises subtypes E1 (Roccaverano strain) and E2 (Seui strain). Both genomes lack the dUTPase subunit of pol gene, the vpr-like accessory gene and the 71 bp U3 repeat but the env hypervariable regions HV1 and HV2 of Seui strain resembles arthritic prototype strains (Reina et al., 2010).

Uracil appears in DNA as a result of dUTP misincorporation or deamination of cytosine (Harris et al., 2003). The dUTPase enzyme acts directly on the pool of nucleotides preventing misincorporation of uracil residues into viral cDNA after RT process by maintaining a low ratio of dUTP:dTTP (Priet et al., 2003). dUTPase is encoded by the pol gene and is a virion-associated protein present in non-primate lentiviruses such as EIAV and CAEV (Chen et al., 2002), however, it is not found in any of the primate lentiviruses (HIV, SIV). Expression of cellular dUTPase is high in dividing undifferentiated cells and low in terminally differentiated non dividing cells (Miller et al., 2000). Moreover, replication of dUTPase-minus CAEV in non dividing cells (e.g. macrophages) leads to a viral loads reduction from 10 to 100 fold compared to the wild type virus (Turelli et al., 1997). Finally, in vivo experiments showed that dUTPase-negative strains produce less severe lesions, usually restricted to the site of injection and reversion of a single point mutation in a dUTPase-negative strain proved to be sufficient to confer a replication-advantage in specific compartments during the course of natural infection, leading to an efficient horizontal transmission (Turelli et al., 1997).

Genome of SRLV can include tat, a retroviral accessory gene encodes a trans-activator peptide upregulating viral expression,

which was designed as vpr-like gene exclusively on the basis of similarity in localization, primary protein structure and function to the HIV-1 vpr. In fact, besides the weak transactivation of viral long terminal repeats (LTR), the CAEV tat (hereafter named vpr-like) increases the viral load and tissue distribution and leads to more severe inflammatory lesions when compared to the vpr-deleted counterpart (Harmache et al., 1995, 1998).

Taken together, these data suggest that both dUTPase and vpr-like retroviral products, although dispensable for viral replication, may confer some evolutionary advantages to SRLVs. The low pathogenic SRLVs analyzed so far are characterized by deletions or mutations in the promoter sequences of LTR, suggesting that these regions are likely to be associated with virulence variation (Barros et al., 2005; Oskarsson et al., 2007). For example, deletion of the 71 bp repeat of CAEV has been associated with a lower cytokine-induced activation of the CAEV promoter (Murphy et al., 2007).

Interestingly, the increasing interest in the last decades for the development of live-attenuated viruses able to induce resistance to superinfection has addressed the specific deletion mutants for the generation of safe and efficacious live vaccine.

In this report, the in vitro properties of Roccaverano and Seui strains, prototypes of the subtypes E1 and E2, have been scrutinized. Surprisingly, even if both dUTPase and vpr-like gene fragments were lost during evolution, subtype E1 was still able to efficiently replicate in monocyte-derived macrophages, while replication was strongly reduced in fibroblast-like cells from canonical target tissues. Roccaverano LTR promoter activity did not show any appreciable difference from that of a conventional genotype B counterpart and was similar in both cellular populations. On the contrary, an entry assay demonstrated different abilities of Roccaverano and Seui strains respectively to penetrate fibroblastic-like cells or blood derived macrophages (BDM). Our data concerning Roccaverano strain provide a new model to understand low pathogenic SRLV and suggest a putative novel target usable for developing safe live-attenuated vaccine.

Results

Virus production

Viral growth curves analysis was carried out to compare three viral strains (CAEV-TO1/89, Roccaverano and Seui). Since Roccaverano strain did not show CPE in any of the selected fibroblastic cell types and syncitium formation in BDM cultures is not necessarily related to SRLV infection (Pisoni et al., 2010), viral production was measured for both B1 and E prototypes by RT activity. Based on standard curves of each strain, the detection limit of RT activity assay was 102 TCID50/ml.

CAEV-TO1/89 as well as Seui strain efficiently replicated in fibroblastic cell types (CP and SM) reaching higher titer comparing to BDM (Wilcoxon test p b 0.01, Figs. 1A and B). In contrast, Roccaverano strain showed different behaviours reaching the highest titer in BDM (Wilcoxon test p b 0.01, Fig. 1C) and no detectable RT activity in fibroblastic cells until 10 days p.i.. However when the latter cell types were sub-cultured at weekly intervals, an increasing in the RT activity was observed, reaching a peak value after 4 weeks (data not shown). We finally noted that Roccaverano viral particles were detectable by RT activity from 5 days p.i., slightly late compared with the other strains. Analyzing viral production of all viral strains in each cell type, CAEV TO1/89 reached higher titer in fibroblastic cells compared to Seui strain (Wilcoxon test p b 0.05), while this difference was not significant in BDM. Roccaverano strain showed higher viral titer in BDM (Fig. 2). No detectable RT activity was obtained in MEC infected by any of the viral strain.

Immunocytochemistry

Specific immunostaining of mouse anti-viral capsid antigen further confirmed the ability of Roccaverano viral strain to replicate in BDM at the highest titer (supplementary file 1). As expected, BrdU incorporation was lower in terminally differentiated BDM compared to fibroblastic cell lines (Fig. 3). Further characterization using NSE and muramidase staining confirmed the macrophage nature of BDM cells (not shown). Finally, co-localization experiments using a macro- phage marker along with a viral antigen, excludes that other, if any, permissive cells may contaminate BDM in vitro during differentiation (i.e. fibroblastic-like cells) (Fig. 4). Even if the Roccaverano strain induced no CPE in foetal fibroblastic cells after four weeks of culture, a positive correlation between RT activity and number of ICC stained cells was recorded.

LTR promoter activity

LTR-U3 sequences from Roccaverano, Seui and CAEV TO1/89 were compared with the CAEV consensus sequence as recently proposed (Murphy et al., 2010). Sequence alignment is reported in Fig. 5 showing the high similarity between genotype E prototypes. We then evaluated the transcriptional activity of two different U3 regions, belonging to Roccaverano and CAEV TO1/89. Both constructs behaved in a similar way and no significant differences were observed between the two promoter regions (Wilcoxon test p N 0.05, Fig. 6). The expression level of the SV40 reporter gene was in agreement with transfection efficiency, being highest in fibroblastic cells. When MEC were used a lower transfection efficiency was recorded as well as SV40 expression level of pCAT control. Nevertheless U3B-pCAT and U3E-pCAT showed a detectable CAT expression, and the comparison between the two promoter activities revealed no significant differ- ences (Wilcoxon test p=0.06, data not shown).

Entry assay

To further evaluate the cell tropism, an entry assay on different target cells (SM, CP, BDM and MEC) was carried out using viral

pseudotypes obtained with the envelope proteins of both Roccaverano and Seui strains. Titers of the same viral pseudotype production were compared on different cell types, for each strain separately (Table 1). The entry assay confirmed the infectivity assay results (Spearman Rho N 0.8, p b 0.05 for both Seui and Roccaverano strains). In particular Seui pseudotype reached a higher titre on fibroblastic cells comparing to BDM, while Roccaverano showed higher pseudoviral titre in BDM compared to fibroblasts (Wilcoxon test p b 0.05 in both cases). How- ever, in contrast with infectivity assay results, pseudoviral titers were obtained on MEC for both pseudotypes (Table 1). Interestingly, AP staining of cells transduced with CAEV-AP/Roccaverano showed just a paranuclear dot-like staining pattern different from those transduced with CAEV-AP/Seui (supplementary file 2).

Discussion

In this study we have demonstrated that the low pathogenic genotype E-Roccaverano strain is able to efficiently replicate in non dividing BDM and poorly in dividing fibroblast-like cells in spite of its characteristic deletions. Since Roccaverano LTR activity was comparable with pathogenic strains from genotype B in all the cells types tested, we focused the hypothesis on the entry steps and its relationship on viral tropism. Indeed, Roccaverano entered preferentially into macrophages but not into fibroblastic cells such as SM or PC compared to Seui or genotype B strains.

Genotype E is highly divergent from the SRLV complete genome sequences known so far. These differences include absence of dUTPase, Vpr-like and lack of a 71 bp repeat within the LTR. According to literature, a virus with these features should be characterized by delayed growth kinetics in dividing cells and by an abrogated replication in non dividing cells, consequent to a genetic drift (Turelli et al., 1996, 1997). Primate lentiviruses (HIV-1) that do not include the dUTPase gene, are able to efficiently replicate in macrophages either by including other nucleotide pool equilibrating mechanisms or by directly removing uracil misincorporation from viral DNA (Priet et al., 2003). The enzymes involved in the base excision repair pathway, which act by specific removal of uracil residues from DNA, belong to the uracil DNA glycosylases

(UNG). Accordingly, HIV-1 vpr accessory protein can modulate virus mutation rate (Mansky, 1996) by docking cellular UNG into viral particles (Mansky et al., 2001). The SRLV vpr-like protein as well as its HIV counterpart induce a specific G2/M arrest of the cell cycle (Villet et al., 2003) followed by apoptosis (Rea-Boutrois et al., 2009). Although the role of CAEV-vpr in the RT fidelity has not been investigated so far, a similar effect cannot be excluded.

Therefore, in our study, it was important to establish the in vitro properties of the Roccaverano strain in several cell systems, including terminally differentiated macrophages. The most striking results obtained in vitro were the different RT activities, being the highest in BDM, reduced or absent in fibroblastic-like cells such as CP and SM or MEC. This pattern of in vitro activity fits well with the biology of this virus in vivo, being infected monocyte/macrophage lineage necessary and sufficient to establish SRLV persistent infection. However, our results are clearly in contrast with previously reported studies (Turelli et al., 1996; Zhang et al., 2003). Animals infected with genotype E develop a robust antibody response, comparable to CAEV-like infected goats (Reina et al., 2009b), suggesting a sufficient expression of viral structural proteins. In co-infected animals, the genotype E-provirus is readily detectable in PBMC even more easily than type B1 provirus (Grego et al., 2007), indicating a potentially higher proviral load in monocytes. Finally, the genotype E could be efficiently transmitted to the offspring by the lactogenic route, as the mammary gland proved to be the only target tissue in which the virus was successfully isolated (Reina et al., 2009a).

Several hypotheses may be considered for efficient replication in non dividing cells. Like in HIV-1, the cellular UNG packaging into virions might involve viral integrase in a vpr-independent mechanism (Willetts et al., 1999). Moreover it has also been confirmed that UNG has a similar role to that played by the dUTPase in preventing the HIV- 1 genetic drift (Priet et al., 2003). Alternatively, an unknown presence of dUTPase activity in caprine BDM cells could be considered, such as transcriptionally active endogenous retroviruses which are usually present in several copies within the small ruminant genome (Hecht et al., 1996; Payne and Elder, 2001). In sheep/goat genome, dUTPase motifs have been found in the endogenous retrovirus, 5′ half of the pro gene and conserved in three endogenous loci compared to the exogenous counterpart (Arnaud et al., 2008; Palmarini et al., 2000). Accordingly, we have detected mRNA species corresponding to the endogenous retrovirus dUTPase (unpublished observation) although effective function is still to be determined. The finding that Roccaverano titer on BDM was significantly higher than those obtained with Seui or CAEV isolates is difficult to explain at this stage. A prolonged survival of infected cells may account for the accumulation of viral particles in the culture medium. In fact, maximum titer of BDM was observed at 10 days p.i. with Roccaverano and after 3–5 days p.i. with other strains. CAEV isolate, inducing apoptosis in infected cells through a mechanism involving VPR could well explain a rapid cell death but Seui strain behaved in a similar way lacking vpr gene.

The relatively low titre of the Roccaverano strain in replicating fibroblastic-like cells was even more surprising. Based on current knowledge, dUTPase and vpr deletions cannot be taken into account for such behaviour. Instead the U3 region of the LTR should be considered. Recently, TNFα and IFNγ have shown to activate the CAEV promoter through mechanisms involving this region (Murphy et al., 2006, 2007), thereby increasing viral load, tissue distribution and

consequently lesion severity. Other enhancer elements such as AP1, AML or CAAT sites could also decrease in number in the absence of this repeat (Barros et al., 2005; Oskarsson et al., 2007). However, our data provide evidence that Roccaverano LTR was as active as the CAEV counterpart or SV 40 promoter, suggesting that the impaired viral production was unlikely due to the absence of transcriptional factors. Moreover, LTR transcriptional activity was similar in SM, CP, MEC and BDM, thus excluding a distinct cell type-related production of transcriptional activators. The U3 regions of Seui and Roccaverano were also very similar except for a genuine AP4 tandem repeat which is present in Seui as well as several CAEV isolates, while a point mutation is present in one of the two repetitions in the Roccaverano. However we showed in previous study a similar transcriptional activity of AP4 and AP4-like sequences (Juganaru et al., 2010). The impaired viral production could be explained evoking a diverse cell- entry ratio in the different cellular populations. This hypothesis has been tested by developing viral pseudotypes and performing entry assays. Viral entry efficiency, expressed in FFU, clearly dem- onstrate that a viral envelope mediates a preferential receptor usage of Roccaverano strain to the macrophage lineage. Sequence comparison between the ENV proteins of Roccaverano and Seui revealed a difference in the amino acid motif of the HV1 and HV2 regions, the latter being closer to that of arthritic isolates. Moreover, while Seui pseudotype conferred to transduced cells the typical AP staining, similarly to various control envelopes (from both CAEV and MVV origin), the atypical cellular localization of AP stain driven by Roccaverano pseudotypes in infected macrophages suggests two non mutually exclusive hypotheses: the Roccaverano virus may penetrate within the cells using an alternative receptor or it may infect a different subpopulation of macrophages, expressing the right receptor. Macrophage activation, driven by different cytokines can polarize into various sub-populations, namely classical-activated, alternative- activated or deactivated macrophages, distinguishable by different expression of cytokine receptor, chemokine receptor and membrane receptor (Gordon, 2003; Martinez et al., 2008). This has severe implications in the HIV-1 viral cycle, since it has been shown that the efficiency in cell entry, transcription and post-transcription of this lentivirus, relies on the macrophage sub-populations to which HIV-1 is in contact with (Herbein and Varin, 2010). In this context, classical activation of macrophages, induced by IFNγ and/or TNFα treatments, may be particularly important in strains bearing conserved GAS and TAS regions within the U3 promoter region. Therefore, it is plausible that the Roccaverano strain, in which both sites are quite polymorphic, takes no substantial advantage to replicate in classically activated macrophages. Instead, it is not surprising that CAEV TO1/89, which had been isolated from synovial membrane of an arthritic goat, displays quite conserved GAS and TAS motifs. Seui strain, having U3 region similar to Roccaverano strain but a putative receptor binding site similar to CAEV isolates may represent a biotype displaying inter- mediate virulence. Once this different biological behavior will be clarified in detail, it will open new approaches towards understanding SRLV derived pathogenesis.

In this study we could not confirm that MEC represent an highly permissive cell lines since neither B1 nor E1 and E2 subtypes showed detectable RT activity after 10 days p.i.. Interestingly Seui pseudotype reached the maximum FFU titer in MEC suggesting that appropriate receptor was expressed. Instead U3 transcriptional activity of both B and E genotypes as well as SV40

positive control was rather low compare to fibroblastic cell lines supporting the idea of a suboptimal viability of MEC at the passage stage in which they have been used.
In conclusion, we have focused our analysis on the biological properties of the new genotype E in vitro, aiming to clarify its pathogenic potential. Since to date genotype E exists in two variants with different biological properties, major deletions in the viral genome may only partially affect virulence, since both subtypes share the same genome organization. The major virulence determinant is likely to be located in the env gene of Roccaverano strain, affecting virus entry and therefore tissue distribution, being reduced or abrogated in some target cells. Moreover, the atypical staining pattern of macrophages infected with Roccaverano pseudotype suggests that an alternative entry pathway could occur, delaying cell death. The high divergence displayed by GAS and TAS sites in the U3 region of both Roccaverano and Seui suggests that classical activation of macrophages is not necessarily the best cellular environment for viral dissemination and tissue damage.

Materials and methods

Viruses and cells

Roccaverano strain (genotype E, subtype E1, GenBank accession number EU293537) was isolated from a seven-year old goat, clinically healthy and with strong antibody response against type E but not against A and B capsid antigens (Reina et al., 2009a).
Seui strain (genotype E, subtype E2, GenBank accession number GQ381130, kindly provided by Dr. Giantonella Puggioni) was isolated in a Sardinian caprine herd from an adult animal suffering from unspecific arthritis, by co-cultivation of BDM with caprine foetal synovial membrane, and fully genetically characterized (Reina et al., 2010).
CAEV field isolate TO1/89 was originally obtained by explantation of synovial membrane from an arthritic goat and partially character- ized in a previous study (Grego et al., 2002). This field isolate was used in in vitro experiments as a conventional genotype B1 isolate.
Synovial membrane (SM) and choroid plexus (CP) cell lines were established as primary cultures from tissue explantation of two caprine foetuses obtained by caesarean section. Diploid cells were maintained in DMEM (Sigma) supplemented with L-glutamine (2 Mm), 10% foetal bovine serum (FBS) and antibiotic/antimycotic solution.
BDM were obtained from uninfected goats belonging to a SRLV- free flock after 9-day culture on Teflon surface or as adherent cells onto 24 well plates (Lechner et al., 1997).
Monocyte/macrophage lineage was first identified through morphological criteria and adhesion strength. Additionally, monocyte–macrophage differentiation was further confirmed by α-naphthyl acetate esterase (NAE) activity and sodium fluoride inhibition of NAE, as well as through immunostaining techniques using VPM32 monoclonal antibody (see below) and muramidase expression.
Milk epithelial cells (MEC), known to be highly permissive to CAEV infection in vitro, were isolated from milk of three uninfected goats belonging to a SRLV-negative flock and processed according to a previously described protocol (Mselli-Lakhal et al., 1999). MEC were grown in RPMI, supplemented with 10% FBS, 2 mM L-glutamine, antibiotic/antimycotic, 10 μg/ml insulin

and 10 ng/ml epithelial growth factor (EGF), and propagated for three passages, to avoid milk mature macrophages carryover.
Foetal cell lines and MEC were stored in liquid nitrogen at low number of passages and used until passage 14 (foetal cells) or be- tween passages 3 and 4 (MEC).
To assess cell cycle progression, a cell proliferation assay was carried out with BDM, MEC, SM and CP cells using BrdU Cell proliferation kit (Calbiochem), following the manufacturer's instructions.
Human HEK 293T cells, which were used for viral pseudotypes production, were grown in the same medium as for caprine foetal cell lines.

Antibodies and immunostaining

Mouse and rabbit antisera were raised against recombinant genotype E capsid antigen and used to detect native viral protein by immunocytochemical (ICC) or immunofluorescent (IF) techniques. Briefly, bacterially expressed viral capsid protein was purified and used to immunize mouse and rabbit using incomplete Freund's adjuvant following standard protocols (Harlow and Lane, 1988). Monoclonal antibody (Mab) VPM32, kindly provided by Dr. Lluis Lujan (University of Zaragoza), had been characterized in previous studies to specifically recognize ovine and caprine macrophage- associated antigen (Alzuherri et al., 1997; Gutierrez and Garcia Marin, 1999; Lujan et al., 1994; Mwangi et al., 1990).
ICC was carried out on 24-well plates following a standard protocol. Briefly, cells were washed with PBS and fixed with methanol:acetone (1:1) for 10 min. An additional incubation with 5% H_2O_2 in methanol was performed for BDM to inactivate endogenous peroxidase activity. Cells were then incubated with mouse anti-capsid serum diluted in PBS with 1.25% casein, for 1 h. After four washes with PBS, a HRP- conjugated goat anti-mouse antibody (KPL), diluted as before, was added. Immunostaining was visualized using DAB–H_2O_2 substrate.
For BDM double staining, standard IF procedures were employed. Briefly, BDM were allowed to differentiate on round coverslips. Cells were washed and fixed as previously described followed by a pre- incubation with PBS-20% goat serum (Sigma) in order to block antibody-Fc binding sites. Primary antibodies (Mab VPM32 and rabbit anti-capsid serum) were diluted in PBS containing 10% goat serum and 1.25% casein and left in place for 1 h at room temperature. After washing, anti-mouse Cy3-conjugated-(Jackson ImmunoResearch Lab- oratories, Inc) and anti-rabbit fluorescein-conjugated antibodies (Vector laboratories), diluted in PBS containing 5% goat serum, were added and incubated as above. After extensive washing, coverslips containing cells were mounted using VECTASHIELD® with DAP (Vector laboratories). For digital imaging a Nikon Video Confocal microscope Eclipse 80i (ViCO Nikon corporation) was used.

Infectivity assay

Each viral strain was propagated in vitro and stored in aliquots at −80 °C until used. One aliquot of each virus was then titrated on appropriate cell culture (SM for CAEV TO1/89 and Seui, and BDM for Roccaverano). Infectivity, expressed as TCID50/ml was evaluated by the presence of cytopathic effect (CPE) (CAEV TO1/89, Seui) or ICC (Roccaverano).
Different cell types (SM, CP, BDM, MEC) were seeded in 24-well culture plates (105 cells/well), and allowed to adhere for 24 h. Cells were washed with PBS and infected with each strain at a M.O.I. of 0.1 for 2 h and then three washes where performed before adding the culture medium.

Mock infected cultures were included in each experiment. A time-course collection, removing an aliquot of different supernatants, was done between 0 and 10–13 days post-infection. All aliquots were centrifuged for 10 min at 500 ×g, 4 °C, and immediately stored at − 80 °C. New viral production was quantified in the supernatant of infected cells measuring the RT activity (HS-Lenti RT Activity kit, Cavidi) following the manufacturer's protocol. In all RT activity experiment five-fold dilutions of each titrated strain were run in the same plate to obtain a strain-specific standard curve (RT activity vs TCID50) thus correlating virus titer with the absorbance of RT activity assay.

Generation of U3 reporter gene constructs

The U3 region of genotype B and E (the latter highly similar between Roccaverano and Seui subtypes), encompassing the whole transcription factors binding sites, was amplified from infected cultures using standard PCR techniques. Amplified products were cloned into pCAT-Basic vector (Promega) and named U3BpCAT and U3EpCAT. Either pCAT-Basic (lacking promoter region) and pCAT- Control (bearing the SV40 promoter), including cloramphenicol acetyltransferase (CAT) as reporter gene, were used for each cell type as negative and positive controls respectively. All plasmids were propagated in E. coli TOP 10 cells (Invitrogen) and plasmid DNA was purified using an endotoxin-free QIAGEN Plasmid Mini Kit.

Transfection of SM, CP and MEC cells was performed in 24 well plates, using 0.9 μg of DNA and Lipofectamine 2000 (Invitrogen) at a ratio of 1:1 (μg DNA:μl Lipofectamine). BDM, were transfected using polyethilenimine microparticles (PEI) conjugated with man- nose, taking advantage of the ability of macrophages to internalize the PEI/mannose/DNA complex via mannose receptor, at a ratio of 1:10 (DNA:PEI). Cells were lysed 48 h after transfection and promoter activity was evaluated by quantifying CAT production by an ELISA procedure (Roche). The promoter expression level for each cell type, derived from four independent experiments, were expressed as per- centage of O.D. value (405 nm) compared to SV40 promoter activity of pCAT control in each cell type. Transfection efficiency was evaluated in situ for each cell type, using pCMV β-gal as a control plasmid and a β-galactosidase expression staining kit (Stratagene).

Production of viral pseudotypes and entry assay

Dr. Isidro Hötzel kindly provided pCAEV-AP and pCMVlcn constructs. The former is a replication-defective CAEV vector, lacking vif protein, and is expressing a termostable human placental alkaline phosphatase (HuPAP) as reporter gene (Hotzel and Cheevers, 2003). The latter is a low-copy-number expression vector (Hotzel and Cheevers, 2001). The pCMVlcn was used as vector to generate the env-expressing constructs of both genotype E variants, namely pCMVRocca and pCMVSeui. The pCMV63, pCMV85/34 and pCMV1514, which code for the full-length env gene of the CAEV-63, MVV-85/34
and MVV-K1514 strains, were again supplied by Dr. Hötzel and in- cluded in our experiment as positive controls of transfection, transduc- tion and AP staining assays (Hotzel and Cheevers, 2002).

All plasmids were propagated in E. coli JM109 cells at 30 °C and purified using endotoxin-free Plasmid Mini and Plasmid Midi kits (Qiagen).

Viral pseudotypes (CAEV-AP/Roccaverano and CAEV-AP/Seui) were produced cotransfecting by electroporation 1×10^6 HEK-293 T cells with 5 μg of pCAEV-AP and 0.5 μg of either

pCMVRocca or pCMVSeui using the Amaxa Nucleofector II device, following manufacturer's protocol from Cell Line Nucleofector Kit V (Lonza). Electroporated cells were seeded in 6-well plates with 2 ml of culture medium and 40h post-transfection the supernatants, containing released replication-defective pseudoviral particles, were collected, clarified by centrifugation for 15 min. at 500 × g, 4 °C and stored at −80°C.

For the entry assay, subconfluent target cells (SM, CP, BDM and MEC) were plated in 24-well culture plates and inoculated, after 24 h, with a tenfold dilution of the above mentioned cell supernatants. Following 2h incubation at room temperature with occasional agitation of the plates, 1 ml of DMEM-10% FBS was added to the cells and incubation proceeded for further 72 h at 37 °C. Foci of cells transduced by pseudoviral particles was evaluated by alkaline phosphatase activity, using the BCIP/NBT staining protocol (http://labs. fhcrc.org/miller/index.html). Pseudotype-viral titers were expressed as focus-forming units (FFU) per milliliter.

Statistical analysis

In order to evaluate the titer of each viral strain in different tissues at different times p.i., linear regression between dilutions of titrated virus and the related absorbances of RT activity was performed. Correlation between dilutions and viral titers was evaluated in logarithmic scale and expressed as R2 values. In the infectivity assay, U3 promoter activity and entry assay, statistical differences were calculated using Kruskal–Wallis Test and Wilcoxon Test either com- paring different virus in the same cell type and different cell types infected by the same viral strain. Finally, correlation between viral titer levels recorded during infectivity and entry assays was evaluated using Spearman's rank correlation test for Seui and Roccaverano viral strains.

All statistical analyses were performed using the R statistical software (R Development Core Team, 2009).

All experiments involving animals used in this study (caprine foetuses, mice and rabbits) were carried out in compliance with the relevant national legislation on experimental animals and animal welfare.

Supplementary data to this article can be found online at doi:10.1016/j.virol.2010.10.031.

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Table 1
Viral pseudotype titer expressed in FFU/ml on different cell lines. Standard error from four replicates is reported within brackets. Maximum titer reached by each viral pseudotypes is reported in bold.

Virus pseudotype	Cell type			
	MSFC	*PCFC*	*BDM*	*MEC*
Seui	1.46×10^3	9.50×10^2	4.63×10^2	$\mathbf{2.10\times10^3}$
	(0.116×10^3)	(0.062×10^3)	(0.116×10^3)	$\mathbf{(0.143\times10^3)}$
Roccaverano	0.18×10^3	0.18×10^3	$\mathbf{1.25\times10^3}$	0.21×10^3
	(0.050×10^3)	(0.037×10^3)	$\mathbf{(0.373\times10^3)}$	(0.064×10^3)

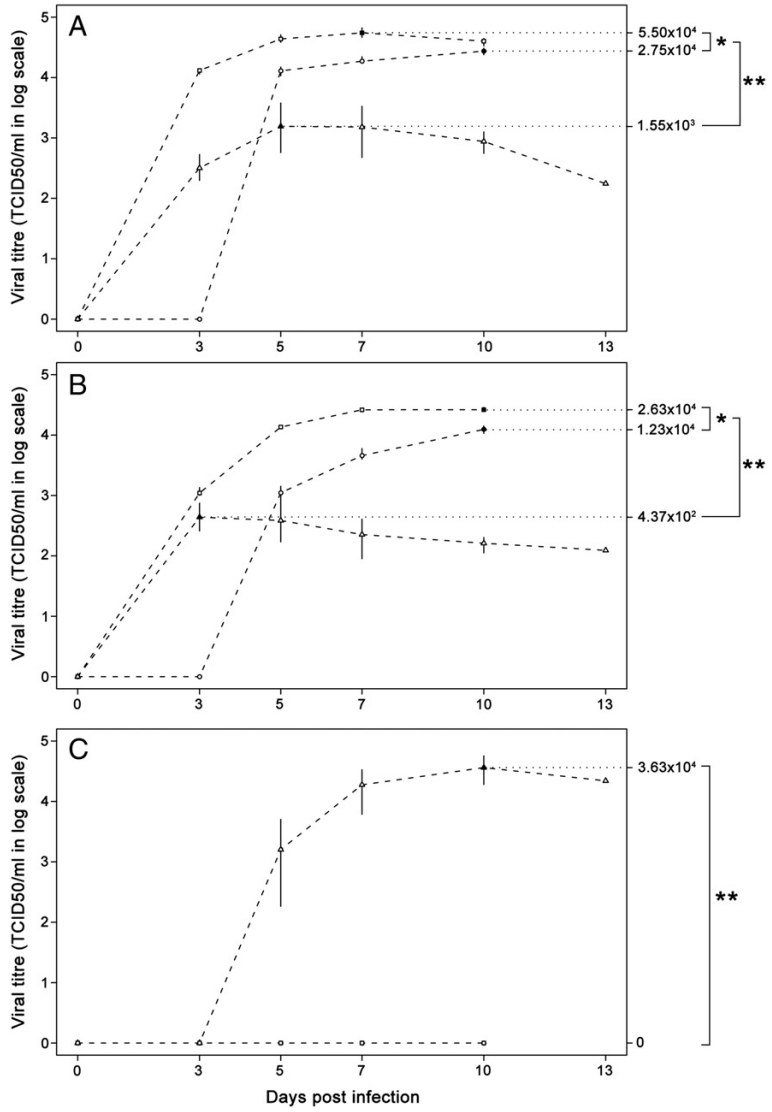

Fig. 1. Viral kinetics. Viral titer kinetics of CAEV *TO1/89* (A), *Seui* (B) and *Roccaverano* (C) viral strains infecting different cell types CP, SM, MEC and BDM. The viral replication was monitored over time between 0 and 10 days for CP, SM and MEC or 0–13 days for BDM and each growth curve indicates the median values of four replicates. Viral replication in the culture supernatants was measured by RT activity assay (405 nm) and expression as TCID50/ml was obtained by strain-specific standard curve (RT activity vs TCID50) included in each experiment. Squares indicate viral titer in SM, circles in CP and triangles in BDM. MEC cell type did not show any detectable viral titer (see text for more details). Vertical bars indicate inter-quartile range of observations. The maximum viral titer reached by each virus in each cell type is represented by black-filled symbols and reported as value on the right y axis. In the right y axis differences between SM and CP and between BDM and fibroblast cells are reported ('*': Wilcoxon test $p<0.05$, '**': Wilcoxon test $p<0.01$).

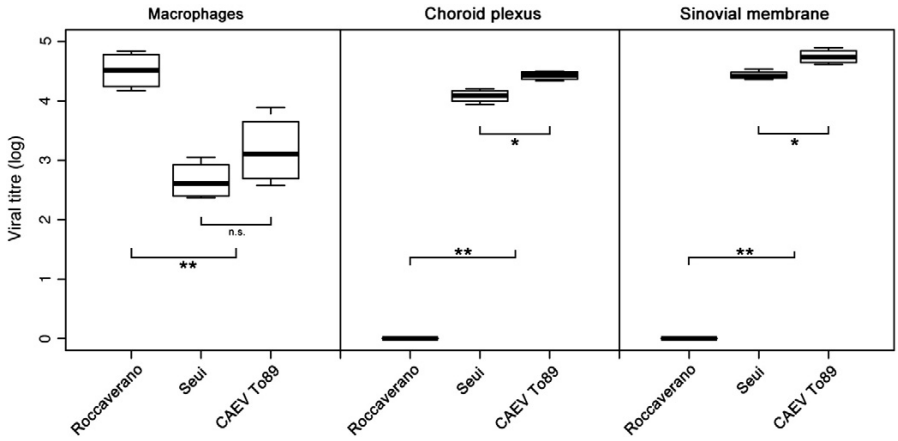

Fig. 2. Viral titer in different cell type. Boxplots indicate the maximum viral titer reached by each viral strain in BDM (A), CP (B) and SM (C). Differences between CAEV-TO89 and SEUI and between Roccaverano and CAEV-TO89/SEUI are reported ('n.s': Wilcoxon test p not significant, '*': Wilcoxon test $p<0.05$, '**': Wilcoxon test $p<0.01$).

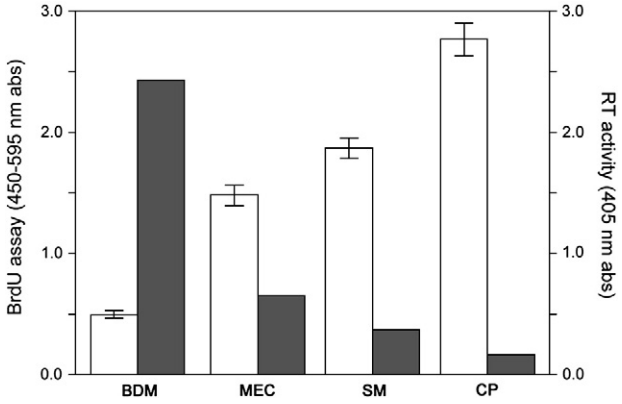

Fig. 3. Cell proliferation assay versus RT activity. Left y axis: Cell proliferation assessed by BrdU incorporation. White bars indicate average delta absorbance (450–595 nm) ± standard deviation (three replica). Right y axis: RT activity (grey bars), of *Roccaverano* strain, obtained in each cell type: BDM, MEC, SM and CP.

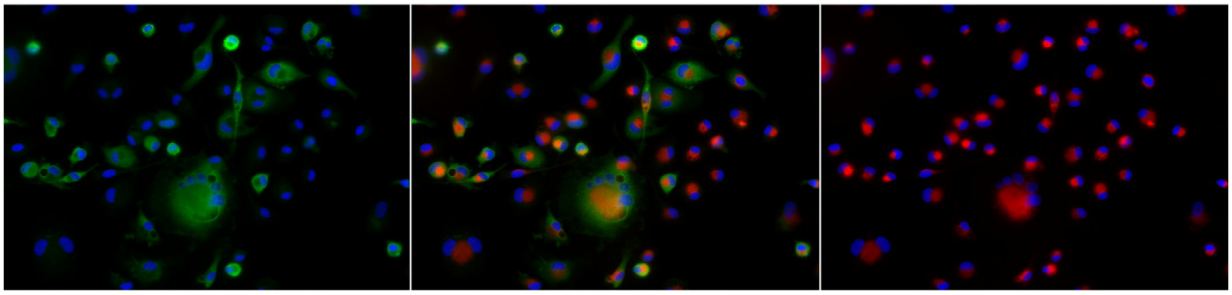

Fig. 4. Staining of BDM after 8 days of infection with *Roccaverano* strain. A: anti P16–P25 genotype E rabbit serum (green). C: anti BDM VPM32 monoclonal antibody (red). B: double staining (merge). Nuclei were stained with DAPI (blue).

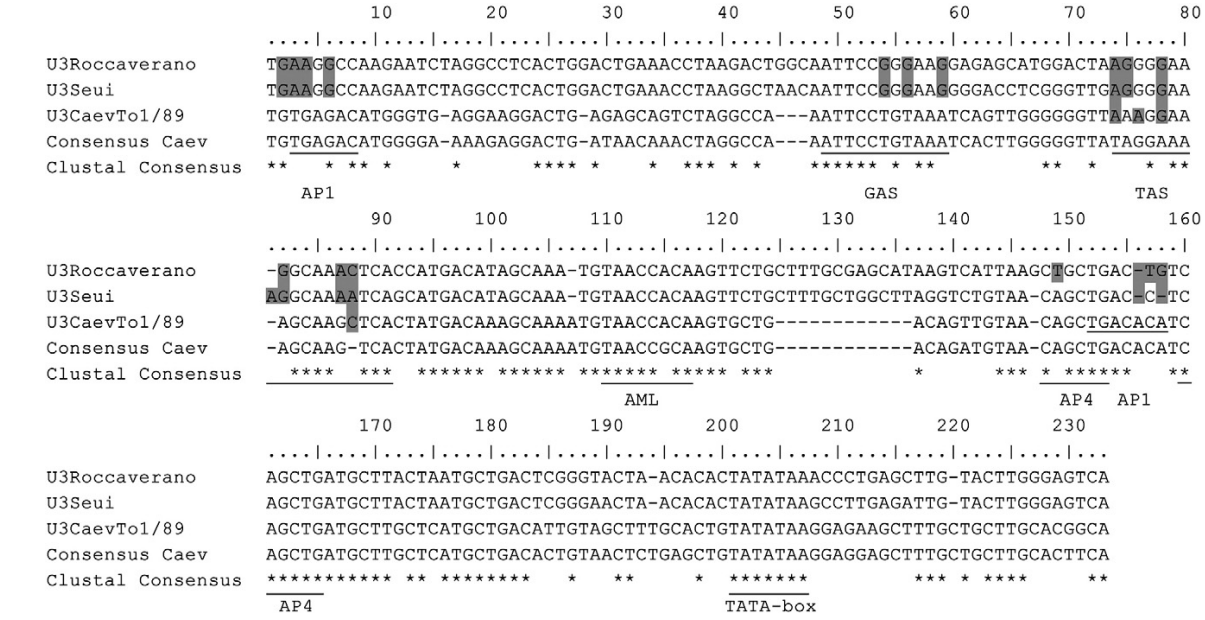

Fig. 5. Alignment of the U3 region sequences of *Roccaverano*, *Seui* and *CAEV-TO1/89* isolates, against consensus CAEV sequence according to reference Murphy 2010. Consensus sequence of each transcriptional factors binding sites and the TATA-box are underlined. Dashes indicate deletions and nucleotides differing from consensus are marked in grey.

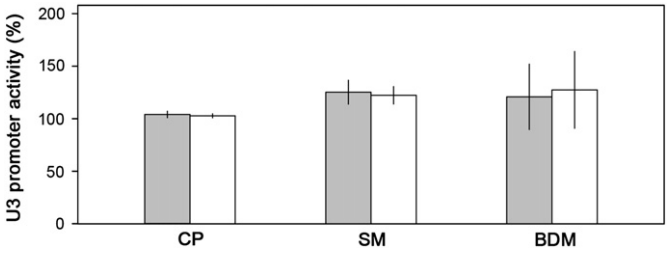

Fig. 6. Transcriptional activities of the U3 region of strain *CAEV-TO1/89* (grey) and strain *Roccaverano* (white) in transfected CP, SM and BDM cells with U3EpCAT and U3BpCAT. pCAT-basic and pCAT-control vectors were used in transfection as negative and positive control respectively for each cell type and also an untreated cell controls (ccNT) was included in each experiment. The expression of CAT was analyzed at 48 h post-transfection, in cell lysates, by ELISA CAT assay and averages from four independent experiments are shown with standard error (vertical bars). The expression level is reported as% to pCAT-Control plasmid (100% reactivity). Results were normalized to the basal activity of the pCAT-Basic (lacking promoter region) in each cell type (not shown).