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Ovine TRIM5α Can Restrict Visna/Maedi Virus

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The restrictive properties of tripartite motif-containing 5 alpha (TRIM5α) from small ruminant species have not been explored. Here, we identify highly similar TRIM5α sequences in sheep and goats. Cells transduced with ovine TRIM5α effectively restricted the lentivirus visna/maedi virus DNA synthesis. Proteasome inhibition in cells transduced with ovine TRIM5α restored restricted viral DNA synthesis, suggesting a conserved mechanism of restriction. Identification of TRIM5α active molecular species may open new prophylactic strategies against lentiviral infections.

Small ruminant lentiviruses (SRLV), including visna/maedi virus (VMV) and caprine encephalitis virus (CAEV), are widespread in sheep and goats, causing a slow progressive disease. Since neither treatment nor efficient vaccines are available, infection is commonly controlled by early diagnosis and culling (23). Recently, the study of host cell restriction factors interfering with the retroviral life cycle, such as the tripartite motif-containing 5 (TRIM5) protein, has gained interest (10, 37). TRIM5 family members bear a RING–B-box–coiled-coil structure consisting of an N-terminal RING domain (with E3 ubiquitin ligase activity), a B-box domain, and a coiled-coil domain (19). The TRIM5α isoform, which is active against retroviruses, contains a C-terminal PRYSPRY domain that binds retroviral capsid CA (12, 20, 35). This interaction, involving amino acid 332 of TRIM5α in humans (15) and 334 in monkeys, may explain the high relative rates of nonsynonymous changes of the primate TRIM5α gene (13). TRIM5α has been described in primates and several mammals (3, 6, 30, 33, 41) but not in sheep or goats, both of which are infected by SRLV, their own lentivirus. This study aimed to identify and characterize the ovine and caprine TRIM5α proteins and explore the possible restrictive role of ovine TRIM5α on VMV infection.

First, we cloned and sequenced ovine and caprine TRIM5α cDNA sequences. For this, total RNA from ovine skin fibroblasts (SF), bronchoalveolar lavage (BAL) fluid, or lung tissue obtained from domestic sheep of the Assaf ($n = 3$), Churra ($n = 2$), and Rasa Aragonesa ($n = 4$) breeds was purified using TRIzol (Invitrogen) passed through RNeasy minikit columns (Qiagen), before being reverse transcribed with SuperScript II (Invitrogen) using an oligo(dT) primer according to the manufacturer's instructions. To clone the caprine counterpart, cDNA from peripheral blood mononuclear cells (PBMC) from goats of the Roccaverano ($n = 1$) and Murciano-Granadina ($n = 2$) breeds was used. These cDNAs were employed as the PCR template using Phusion high-fidelity DNA polymerase (Finnzymes) with the forward primer TrimEXNFw (5′-TGCA CCTCGAGATGGCTTCAGGAATCCTG-3′, XhoI site underlined) and the reverse primer PJ2 (5′-GATCCGGGCCCTCAAC AGCTTGGTGAGC-3′, ApaI site underlined) following standard thermal profiles. Amplified products were cloned into the TOPO Blunt vector (Invitrogen) as a shuttle/sequencing vector, yielding a total of 12 ovine and 5 caprine independent sequences. Four ovine sequences were obtained at least twice and were aligned with previously described TRIM5α sequences (ClustalX and PHYLIP: Phylogeny Inference Package version 3.5c), revealing a conserved structure across species. Analysis of six clones from SF of one Rasa Aragonesa sheep revealed the presence of only two TRIM5α amino acid sequences (named Ov1 and Ov2), suggesting that these sequences are encoded by a single heterozygous gene. The sequences differed only at a single residue (39) of the PRYSPRY-domain V1 region. Greater levels of amino acid diversity were found in additional sheep and goat sequences (Fig. 1). To examine sequence diversity, phylogenetic trees were produced by the neighbor-joining method with Kimura's correction using 1,000 bootstrap confidence limits. Results with over 950 bootstraps were considered highly likely. As expected, ovine and caprine sequences were closely related, followed by bovine sequences (Table 1), forming a nonprimate TRIM5α cluster (Fig. 2). Comparison of these sequences revealed greater variation between caprine and ovine TRIM5α proteins than between ovine sequences (Table 1; Fig. 1A), with the PRYSPRY being the most variable domain. Such variation was higher than expected given that sheep and goats diverged 6 million years ago (16), whereas humans and chimpanzees, which encode more highly related TRIM5α sequences, diverged 7 million years ago (5). The close relatedness between sheep and goats is consistent with the ability of sheep (VMV) and goat (CAEV) lentiviruses to infect both ruminant species (8, 32). The high variability of both PRYSPRY (6, 34; this work) and CA of SRLV (7, 26) may account for the evolution of both virus and host, involving TRIM5α and CA interactions, as described for primate lentiviruses (11, 28, 34, 38). Natural selection in ovine and caprine sequences was determined by estimating ω (ratio of the rate of nonsynonymous substitutions, dN, to synonymous substitutions,

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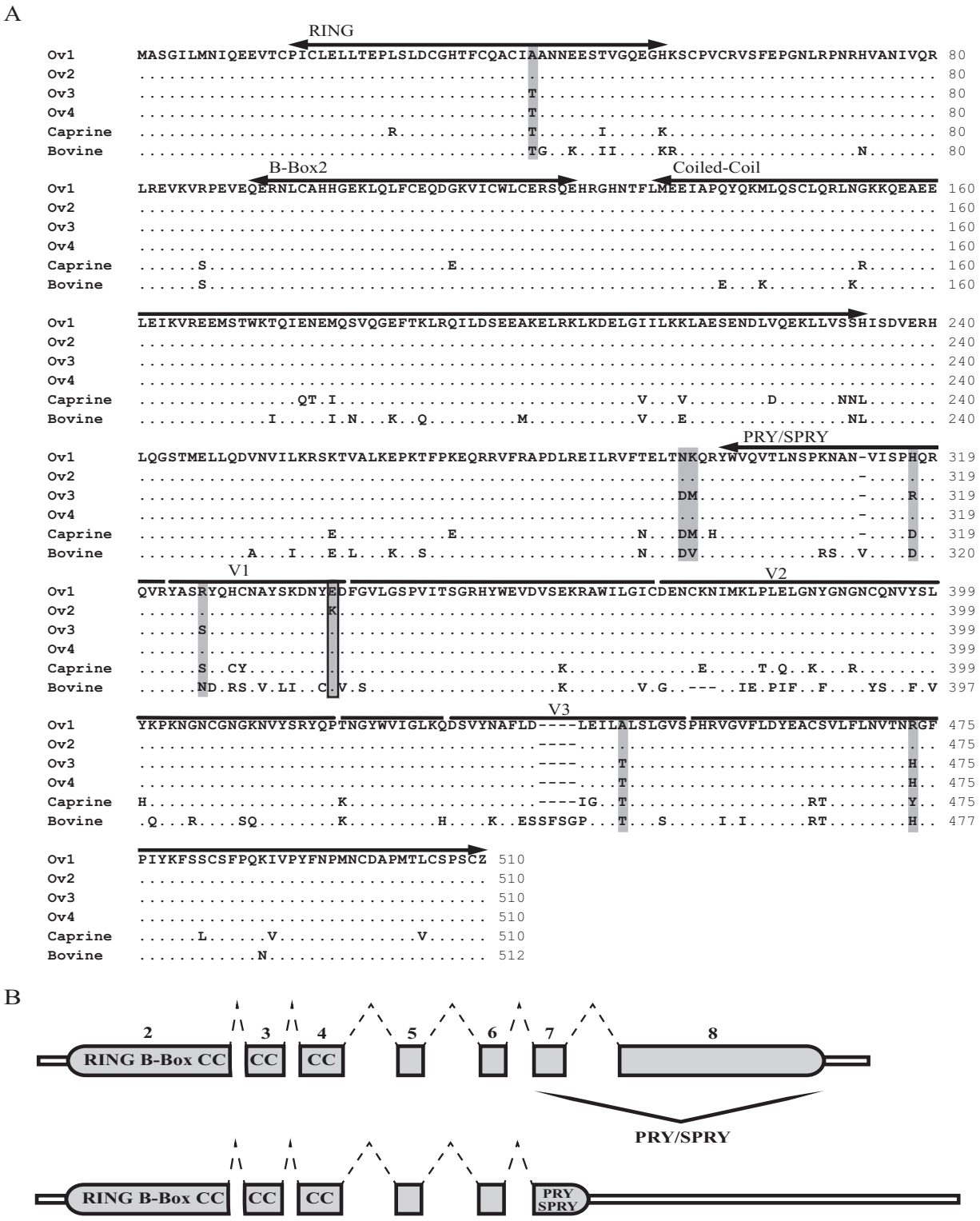

FIG 1 TRIM5α amino acid sequences from ruminants. (A) Alignment of TRIM5α amino acid sequences from ovine (Ov1 to Ov4), caprine, and bovine species. Ov1 and Ov2 were amplified from skin fibroblasts of one seronegative Rasa Aragonesa breed sheep. Ov3 and Ov4 were amplified from bronchoalveolar lavage fluid cDNA from a seropositive Rasa Aragonesa sheep. RING, B-box-2, coiled-coil, and PRYSPRY domains are shown, and the variable segments (V1, V2, and V3) of this domain are outlined. Amino acid differences between ovine sequences (Ov1 to Ov4), including those corresponding to V1 residue 339 among others, are highlighted in gray. Accession numbers (GenBank) are as follows: Ov1 to Ov4, JN835300 to JN835303; caprine, JQ582845; bovine, DQ380509. (B) Structure of the ovine TRIM5: full-length isoform (top) and an alternatively spliced form (bottom).

TABLE 1 Sequence identity matrix[a]

	% Similarity with sequence type									
	Ov2		Ov3		Ov4		Caprine		Bovine	
Sequence type	T5	PRYSPRY	T5	PRYSPRY	T5	PRYSPRY	T5	PRYSPRY	T5	PRYSPRY
Ov1	99	99	98	98	99	99	91	89	84	76
Ov2			98	97	99	98	91	89	83	75
Ov3					99	99	92	90	84	77
Ov4							92	90	84	77
Caprine									84	75

[a] Sequence identity matrix for the complete coding sequence (CDS) of the TRIM5α protein (T5) and its PRYSPRY domain, using the ovine (Ov1, Ov2, Ov3, and Ov4) and caprine amino acid sequences obtained and a bovine sequence (DQ380509) available at GenBank.

dS), using three methods: single-likelihood ancestor counting (SLAC), fixed effects likelihood (FEL), and random effects likelihood (REL) implemented in the Datamonkey webserver (21). The existence of strong positive selection observed when comparing other species (6, 17, 29, 34) was not observed in sheep-versus-goat comparisons ($P \geq 0.064$), consistent with viral transmission between these species.

Additional RNA species (6 from a total of 3 sheep) were identified that had a stop codon at residue 347 compared to the full ovine protein TRIM5α (OvT5α), resulting in a reduced number of exons and elongated noncoding sequences at the 3′ end, a structure similar to that of human TRIM5γ (2) (Fig. 1B). No splicing donor/acceptor consensus sequences were found in the shortened TRIM5α sequences under study, suggesting that truncated proteins are indeed splice variants of TRIM5 and that splicing of these forms is conserved between primates and ruminants. Numerous isoforms that are shorter than the antiviral TRIM5α exist in humans (2) and macaques (4). Some of these lack the PRYSPRY domain and are therefore unrestrictive. Like human TRIM5γ (36), the short ovine isoform is likely to act as a dominant negative through lack of a viral binding PRYSPRY domain (2).

To characterize restriction by OvT5, sequences Ov1, Ov2 (both from SF of a seronegative Rasa Aragonesa sheep from a seropositive flock), and Ov4 (obtained from BAL fluid cells of a Rasa Aragonesa seropositive sheep affected with pneumonia) were cloned into the gammaretroviral expression vector pCNCR-HA, using XhoI/ApaI restriction sites. The vector contains the LTR of Moloney murine leukemia virus (MLV), driving expression of an N-terminal hemagglutinin (HA)-tagged OvT5α protein and the gene for the red fluorescent protein (RFP). The resulting vector (pCTCR-HAOvT5) was packaged into vesicular stomatitis virus G envelope protein (VSV-G)-pseudotyped MLV cores by cotransfection of 293T cells as described previously (3). Culture supernatants containing MLV virions encoding OvT5 were used to transduce the *Mus dunni* tail fibroblast (MDTF) cell line, and HA-tagged OvT5 stably expressing cells were obtained. Cells transduced with empty pCNCR-HA were used as controls. Single cell clones were isolated by limiting dilution, identified by red fluorescence microscopy, expanded, and checked for expression of TRIM5α proteins by Western blotting (WB) using anti-HA antibodies and quantitative reverse transcriptase PCR (RT-PCR) with forward (qPCR3T5Fw: 5′-TTCCTAGACTATGAGGCTTGCTCTG T-3′) and reverse (qPCR3T5Rv: 5′-TTCTGAGGAAAGGAACAT GAAGAGA-3′) primers, designed within the PRYSPRY region. β-Actin RT-PCR allowed relative quantification using the primers described (24).

Transduced MDTF cells expressed OvT5-HA according to the results of WB (Fig. 3B) and RT-PCR (not shown). These OvT5-expressing clones were subjected to infection by VMV strain Ev1 (27) to study restriction. The dose was determined by titrating Ev1 by inoculation of 10-fold serial dilutions onto ovine SF and visualization of the cytopathic effect by microscopy after 7 days. Titers, calculated by the Reed-Muench method (22), were expressed as 50% tissue culture infectious doses ($TCID_{50}$) per ml. Cells were infected at an apparent multiplicity of infection (MOI) of 0.2, and 16 h later MDTF total DNA was purified using a QIAamp DNA minikit (Qiagen). TaqMan quantitative PCR (qPCR) was used to measure viral DNA synthesis using a plasmid standard curve as described previously (9). Strain Ev1 entered and was reverse transcribed in the MDTF cells. Viral DNA was detected by qPCR (mean copy number/100 ng DNA when infecting at 0.2 $TCID_{50}$/cell, 9.9×10^2), and viral transcripts were produced according to RT-PCR (not shown). Thus, heterologous MDTF cells were suitable for assessing OvT5-mediated restriction, even though infection was not productive since supernatants had no RT activity up to day 20 postinoculation (not shown). Measurement of viral DNA synthesis indicated that MDTFs transduced with OvT5α were less permissive to reverse transcription than control MDTFs ($P < 0.05$). We conclude that OvT5α was able to significantly restrict VMV infectivity. TRIM5α Ov1 and Ov2 were able to restrict Ev1 whereas TRIM5α Ov4 was not, despite strong expression detected by immunoblotting (Fig. 3A and B). In the TRIM5α protein of humans and simians, a single amino acid substitution at position 332 or 334 abrogates TRIM5α-mediated restriction of particular viruses due to its essential role in viral recognition (13). Specifically, any non-positively charged amino acid at that position, which belongs to a "patch" of positively selected positions, improves CA binding (15). Surprisingly, Ov1 and Ov2 had either a positively charged amino acid (K) or a negative residue (E) at this position (Fig. 1A), but both showed a similar restriction of Ev1 in MDTF cells. Due to the poor alignment of this highly variable region, it is difficult to be sure that this amino acid is analogous to primate TRIM5 position 339, but it is certainly very close and putatively present on the surface of the protein in the highly variable loop that is most important for determining TRIM5α specificity. Recent studies highlight the importance of domains other than PRYSPRY (18). Significantly, Ov4 had differences in the RING and PRYSPRY domains compared with Ov1 and Ov2

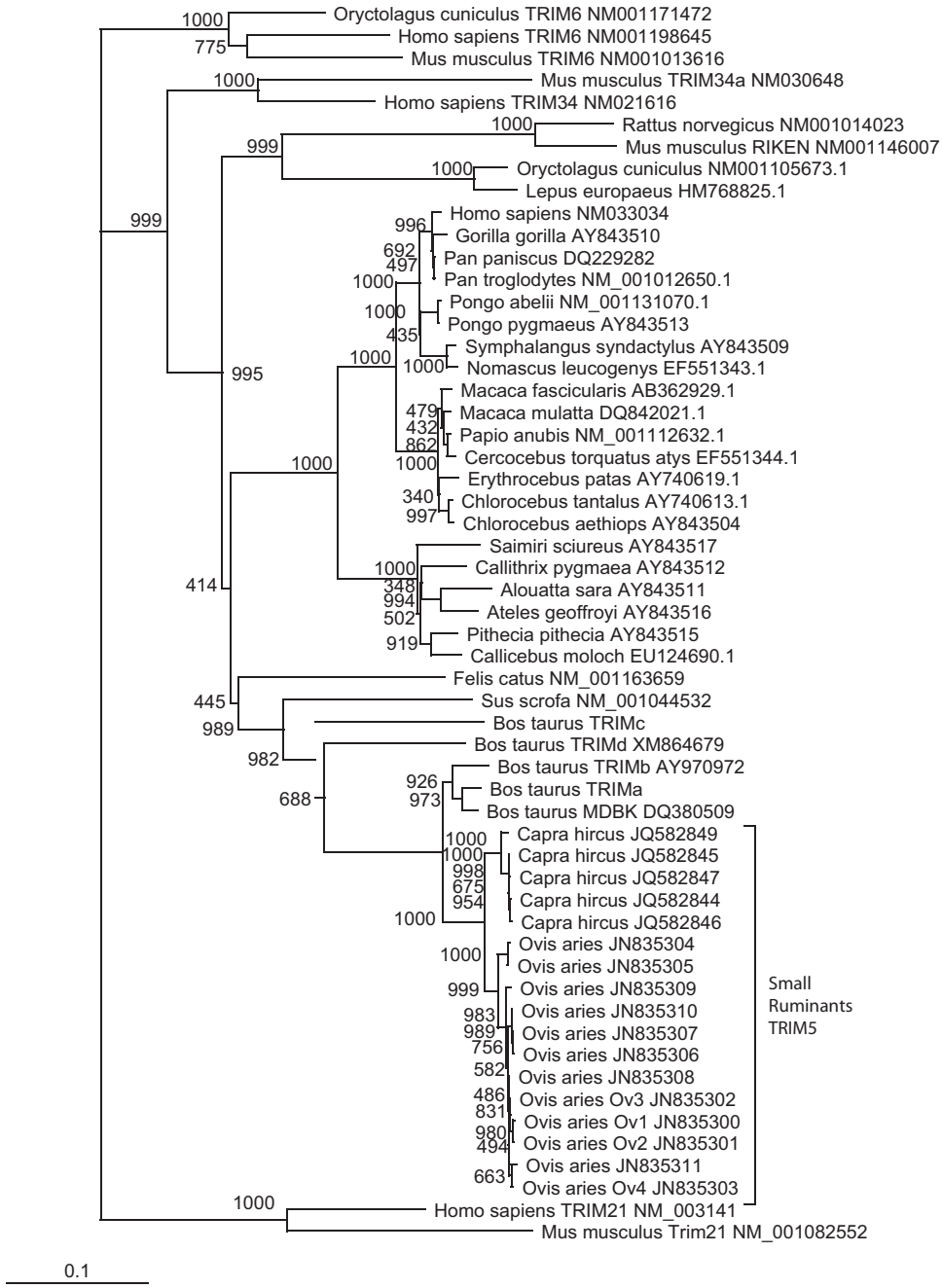

FIG 2 Phylogenetic tree of TRIM nucleotide sequences of different species. The tree shows that all the sheep and goat TRIM5 sequences described in this work are TRIM5α orthologues. GenBank accession numbers shown are for sequences from the following sources: JN835300 and JN835301, Rasa Aragonesa skin fibroblasts; JN835302 and JN835303, Rasa Aragonesa bronchoalveolar lavage fluid; JN835304, Rasa Aragonesa lung tissue; JN835305, lung tissue of two Rasa Aragonesa and two Assaf sheep; JN835306, lung tissue of two Assaf and one Churra sheep; JN835307, Assaf lung tissue; JN835308, Rasa Aragonesa lung tissue; JN835309, Rasa Aragonesa lung tissue; JN835310, Churra lung tissue; JN835311, lung tissue of one Assaf and one Churra sheep; JQ582845, peripheral blood mononuclear cells of a Roccaverano goat; JQ582846 to JQ582848, peripheral blood mononuclear cells of a Murciano-Granadina goat; JQ582849, peripheral blood mononuclear cells of a Murciano-Granadina goat.

and may have lost restrictive activity against VMV. Indeed, Ov4 was obtained from an infected sheep, consistent with a permissive TRIM5 genotype.

In addition, we tested the restrictive role of OvT5 against VSV-G-pseudotyped HIV-2 viral vectors encoding green fluorescent protein (GFP), prepared by Fugene-6 transfection of 293T cells as described previously (10). We infected OvT5-MDTF cells and quantified infection at 48 h by measuring GFP expression using flow cytometry (BD FACScalibur). MDTFs showed decreased levels of HIV-2 infection when expressing Ov2 (Fig. 3A), strongly suggesting a role for OvT5 in protecting sheep from HIV-2 infection.

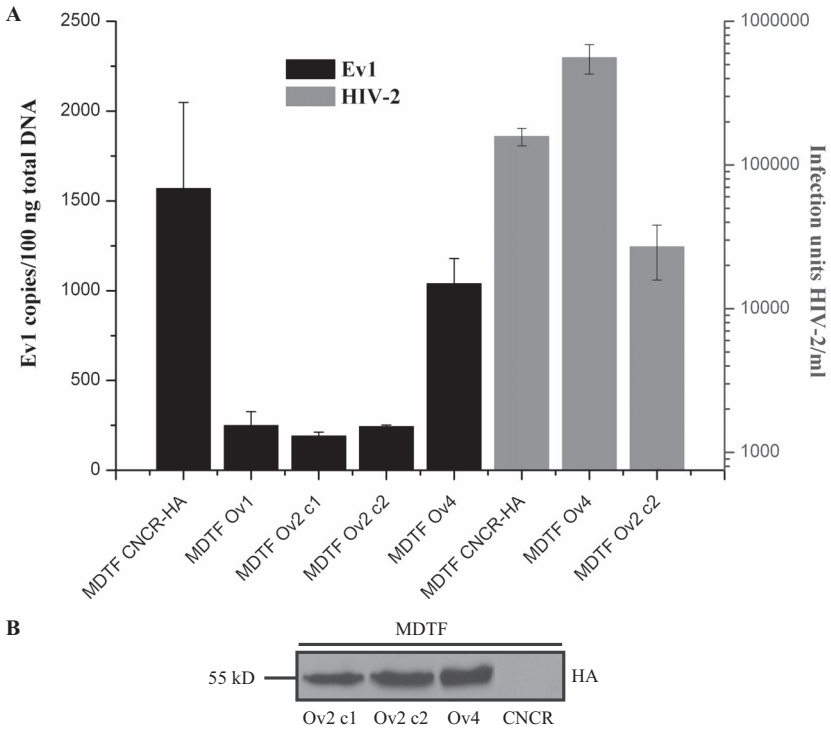

FIG 3 Ovine TRIM5α (OvT5α)-mediated restriction. (A) MDTF cells expressing OvT5α were infected with GFP-expressing HIV-2 VSV-G pseudotyped lentiviral vector or VMV strain Ev1. Data represent viral DNA copy numbers (mean values ± standard error) per 100 ng of total DNA obtained by qPCR using Ev1-specific primers/probe (9) or infectious units per ml in the case of HIV-2. Cells transduced with pCNCR-HA were used as negative controls. Three independent experiments were performed. (B) Western blot illustrating the expression of different HA-OvT5 sequences in MDTF cells (transduced pCTCR-HA-OvT5 or empty vector CNCR), using anti-HA-tag antibodies.

Since the proteasome has been shown to be involved in TRIM5α restriction in other species (1, 25, 40), we inhibited the proteasome and examined OvT5-mediated restriction of VMV. MDTF cells expressing TRIM5α Ov2 or empty vector were treated with prewarmed (37°C) proteasome inhibitor MG132 (Sigma-Aldrich) at a final concentration of 25 μM for 1 h before infection with Ev1 at an MOI of 0.2. After 16 h of infection, viral DNA was quantified in duplicate by qPCR as described above, and the experiment was repeated three times. The results indicated that viral DNA was significantly increased ($P < 0.01$) in proteasome-inhibited MDTF cells expressing a restrictive OvT5 compared to untreated cells. Either treated or untreated cells transduced with an empty vector showed no viral restriction, having similar levels of viral DNA (Fig. 4). The involvement of the proteasome in OvT5 restriction is in line with findings on other lentiviruses (1, 40).

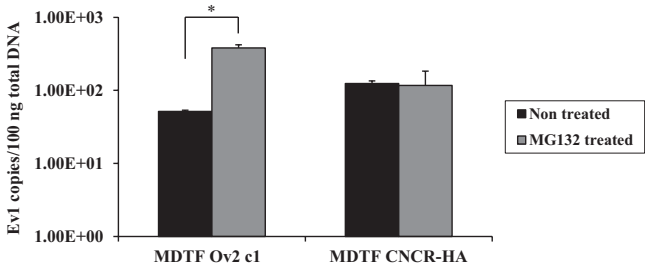

FIG 4 Effect of proteasome inhibition on VMV DNA levels in ovine OvT5α-expressing cells. MDTF cells expressing restrictive OvT5α Ov2 or transduced with an empty CNCR-HA vector were treated for 1 h with proteasome inhibitor MG132 (untreated cells were used as a control) before infection with VMV strain Ev1, and viral DNA was measured 16 h after infection by qPCR using Ev1-specific primers/probe. Data represent viral DNA copy numbers (mean ± standard error) per 100 ng of total DNA. Three independent experiments were performed.

TRIM5α has been characterized mostly in the context of restricting heterologous viruses, but as shown here homologous virus restriction may also take place upon overexpression of TRIM5α (14, 42). This suggests that differences in TRIM5 expression levels as well as intrinsic antiviral specificity may account for differences in permissiveness to infection between individuals (31, 42). Importantly, our study suggests functional differences between polymorphic ovine TRIM5α variants to restrict both heterologous (HIV-2) and homologous (VMV) viruses. A better understanding of these differences could eventually be used to design SRLV control strategies such as identification and selective breeding of animals that are less permissive to infection, thus avoiding culling and helping to reduce viral load and therefore disease development.

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this work were deposited in GenBank under accession numbers JN835300 to JN835311 (ovine) and JQ582845 to JQ582849 (caprine).

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