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Diversity of caprine arthritis–encephalitis virus promoters isolated from goat milk and passaged in vitro

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ABSTRACT

Transcriptional regulation in retroviruses resides in the U3 region of the proviral long terminal repeat (LTR). Transcription binding sites (TBS) in the U3 region of proviral sequences derived from the milk of 17 goats infected with caprine arthritis–encephalitis virus (CAEV) were analysed by nested PCR and sequencing. U3 sequences shared a high degree of homology (86–99%) and were closely related to isolates previously ascribed to small ruminant lentivirus subtype B1. Multiple putative AP-1, AP-4, Ets-1, Stat-1 and TATA binding protein (TBP) sites were highly conserved (>85% of isolates), as were single AML(vis), GAS, IRF-1, NFAT and TAS sites. A 10 nucleotide insertion of undetermined relevance was identified in the U3 region of two isolates. To study the stability of TBS within the CAEV U3 region through in vitro passage, milk-derived isolates of CAEV from three infected dams were cultured in goat synovial membrane (GSM) cells; in one isolate the viral U3 region was completely stable during in vitro passage, in a second isolate the viral U3 region accumulated multiple deletions, single nucleotide polymorphisms and insertions, while a third isolate had an intermediate degree of promoter stability. Promoter mutations arising during in vitro passage did not affect most of the conserved putative TBS identified in CAEV.

Introduction

Caprine arthritis–encephalitis virus (CAEV) and maedi-visna virus (MVV) are small ruminant lentiviruses (SRLVs) affecting goats and sheep, respectively (Leroux et al., 2010). CAEV is transmitted horizontally when neonatal kids ingest CAEV-positive colostrum or milk and also can be transmitted by respiratory aerosols (Blacklaws, 2012). Goats remain infected for life and clinical signs often are not evident until months or years after initial infection; some infected animals do not exhibit clinical disease (Trujillo et al., 2004). Infection with CAEV is associated with inflammatory disease localised to the synovial joints, central nervous system, mammary gland and/or lungs. CAEV infection of the mammary gland can result in mastitis, which may be subclinical (Lerondelle et al., 1989). The absence of clinical mastitis and occult transmission of the virus through milk or colostrum perpetuates infection in goat herds.

Integrated CAE provirus may remain latent, with little or no transcriptional expression (Angelopoulou et al., 2008; Murphy et al., 2010). Specific cellular transcription factors activate viral gene expression by interacting with proviral DNA sequences in the long terminal repeat (LTR), referred to as transcription binding sites (TBS) (Clements and Zink, 1996; Pépin et al., 1998). The LTR is divided into three regions: U3 (which contains the viral promoter), R and U5. TBS in the CAEV U3 region include activator protein (AP)-1, AP-4, interferon (IFN) regulatory factor (IRF)-1, γ-interferon activated site (GAS), CCAAT, the TATA box and acute myeloid protein of visna virus or AML(vis) (Hess et al., 1986; Speck and Baltimore, 1987; Tong-Starksen et al., 1996; Sepp and Tong-Starksen, 1997; Angelopoulou et al., 2008; Glaria et al., 2009; Murphy et al., 2010; Ramírez et al., 2011). Several studies have suggested that the AP-1, AP-4 and AML(vis) motifs are required for SRLV transcriptional activity (Gabuzda et al., 1989; Gdovin and Clements, 1992; Campbell and Avery, 1996; Sutton et al., 1997; Barros et al., 2005; Juganaru et al., 2010), although these findings were not supported by the study of Murphy et al. (2006).

Inflammatory processes induced by CAEV at different sites are associated with the production of cytokines, which may regulate viral transcription through cellular signalling pathways mediated by TBS in the LTR (Lechner et al., 1997a). The pro-inflammatory cytokines IFN-γ and tumour necrosis factor (TNF)-α, which are increased in joint lesions of goats with CAEV-induced arthritis (Lechner et al., 1997b), activate viral transcription through the γ-IFN activated site (GAS) and TNF-activated site (TAS),

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respectively, in the CAEV U3 region (Tong-Starksen et al., 1996; Murphy et al., 2006). IFN-γ activates CAEV transcription through a JAK-STAT signalling mechanism, by which phosphorylated Stat-1 dimers bind to the CAEV GAS (Sepp and Tong-Starksen, 1997).

CAEV is transmitted primarily through suckling and mammary gland associated virus serves as the main route of dam-to-kid transmission. The combinatorial array of TBS in the U3 region of mammary gland-associated CAEV may play a role in determining disease outcome for the infected kid. The aim of this study was to explore the diversity, conservation and stability of promoter-based TBS in viral isolates derived from the milk of CAEV-infected goats.

Materials and methods

Animals and milk samples

All goats included in the study were located in farms/flocks in Central California. Serology for CAEV was performed with the Caprine Arthritis–Encephalitis Virus Antibody Test Kit, cELISA (VMRD). Caprine milk was obtained aseptically from 16 CAEV seropositive goats (Alpine: $n = 3$; La Mancha: $n = 5$; Nubian: $n = 2$; Saanen: $n = 3$; Toggenburg: $n = 3$) in their second to fourth lactation. Milk samples (1.5 mL each) were stored at −20 °C until analysed. Fresh milk (~60 mL) from six additional goats (four CAEV-seropositive and two seronegative animals) was analysed within 12 h after collection (Table 1). One of these goats (S-191) had slightly enlarged carpi, but none had indurative mastitis or other clinical signs attributable to CAEV infection (Table 1).

Milk was centrifuged at 2200 *g* for 5 min, the fat layer was removed with a sterile pipette tip and the resulting milk-derived cell pellet was washed three times with cold phosphate-buffered saline (PBS). After the final wash, the cell pellet was resuspended in 10 mL PBS and 500 μL of the resuspended cells were removed for genomic DNA extraction. The remaining cells from each sample were divided into two lots for cell culture.

Cell culture

Goat synovial membrane (GSM) cells were derived and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal bovine serum (FBS) as described by Klevjer-Anderson and Cheevers (1981). Caprine milk-derived cells were grown in 25 cm^2 flasks (Corning), each containing 5 mL RPMI 1640 (Gibco) supplemented with 12% FBS, 4 μL/L 2-mercaptoethanol, 5 μg/mL concanavalin A (ConA, ThermoFisher Scientific), 0.5 μg/mL phorbol myristate acetate (PMA, Sigma-Aldrich) and 1% or 2% antibiotic solution (100 or 200 U/L penicillin and 100 or 200 μg/L streptomycin, respectively; Gibco).

Caprine milk-derived cells were cultured for either 3 or 4 days (in medium containing 2% or 1% antibiotic solution, respectively), centrifuged at 300 *g* (Sorvall 7RT), resuspended in 5 mL DMEM and co-cultured with 90% confluent GSM cells. Supernatants were harvested after 14 d (passage 1), clarified by centrifugation at 1100 *g* (Sorvall RT7) for 20 min and stored at −80 °C in 1 mL aliquots. A 1 mL aliquot from each culture was used to infect 25 cm^2 flasks of semi-confluent GSM cells from which the culture media had been aspirated. After a 2 h incubation period, 5 mL fresh DMEM medium was added to each flask. Supernatants were harvested after culturing for 14 d (passage 2) and processed as for passage 1. On day 14 of passages 1 and 2, a sterile cell scraper (BD Falcon) was used to harvest approximately 25% of the attached GSM cells from each flask. Detached cells were resuspended in 1 mL PBS and transferred to 1.5 mL tubes for genomic DNA extraction.

Table 1
Signalment of goats from which fresh milk was collected for isolation of caprine arthritis encephalitis virus (CAEV).

Identification	CAEV serological status	Breed	Age (years)	Clinical condition
L-11	Positive	La Mancha	6	Clinically healthy
L-217	Positive	La Mancha	5	Clinically healthy
S-191	Positive	Saanen	3	Slightly enlarged carpi
N-214	Positive	Nubian	3	Clinically healthy
93	Negative	Toggenburg	2	Clinically healthy
95	Negative	Toggenburg	2	Clinically healthy

Nested PCR and analysis of sequenced products

Genomic DNA was extracted with the QIAamp DNA Mini Kit (Qiagen) and stored at −20 °C. Nested PCR using oligonucleotide primer pair R_{rev} and Rev_{for}, followed by the nested PCR primer pair $CAE_{Nest\ LTRfor}$ and $CAE_{Nest\ LTRrev}$, was used to amplify the 3′ U3 region (Murphy et al., 2012). Reactions were performed in a total volume of 100 μL containing 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dNTP), 0.4 μM each primer, and 0.0125 U/μL *Taq* DNA polymerase (AmpliTaq, Invitrogen). Ten microliters of extracted genomic DNA was used for the first reaction and 7 μL of the first reaction was added to the second amplification reaction. Amplification conditions were 95 °C for 2 min, followed by 50 cycles of 95 °C for 15 s, 56 °C for 30 s and 72 °C for 30 s, then 72 °C for 5 min. After gel electrophoresis, selected PCR products were column purified (Amicon 100 K, Millipore) and both strands were sequenced.

Sequences were deposited in GenBank under accession numbers JQ958579–JQ958595 (Fig. 1). CAEV U3 nucleotide (nt) sequences of these and previously reported sequences from GenBank were aligned and phylogenetic trees were obtained using ClustalW2 (Larkin et al., 2007), Vector NTI (Invitrogen) and TreeView (Page, 1996) (see Appendix A: Supplementary material). The Jaspar algorithm (Bryne et al., 2008), with a relative profile score threshold of 80%, was used to search for AP-1, CEBP/C, CCAAT box, CREB, Ets-1, IRF-1, nuclear factor of activated T cells (NFAT), nuclear factor κB (NF-κB), Sp-1, Stat-1 and TATA binding protein (TBP) sites. AML(vis), AP-4, GAS, glucocorticoid responsive element (GRE), IFN-stimulated responsive element (ISRE) and TAS sites were searched for manually. Passaged viral isolates were named according to the following system: cell line/code number of the goat.percentage of antibiotic solution used initially for culturing milk-derived cells_number of passages (Fig. 2a).

Results

Transcription binding sites in caprine arthritis–encephalitis virus U3 sequences from caprine milk-derived cells

CAEV U3 sequences were amplified by nested PCR from 13/16 (81.3%) frozen milk samples and all four CAEV-seropositive fresh milk samples (Fig. 3). Sixteen sequences were obtained from clinically healthy goats and one sequence was obtained from the goat (S-191) with slightly enlarged carpi (Table 1). No amplicons were detected in samples from the CAEV-seronegative goats. A high degree of homology within the U3 region was shared by all amplicons (86–99% similarity in the U3 region) and most of the isolates clustered tightly (Fig. 4).

Multiple putative AP-1 and Ets-1 sites were located using the Jaspar algorithm; the number of AP-1 sites ranged from three (isolate 363) to seven (isolates 360 and N-214) and the number of Ets-1 sites ranged from six (isolates L-11 and 354) to nine (isolates 360, 363 and S-191). Four AP-1, two AP-4, eight Ets-1, two Stat-1 and three TBP (TATA binding protein) sites were highly conserved, as were single AML(vis), GAS, IRF-1, NFAT and TAS sites (Fig. 1). Other TBS within the U3 region (five AP-1, CEBP/C, CREB, six Ets-1, GRE, ISRE, one of the IRF-1, NFAT, NF-κB and Sp-1) were conserved in 0–35% of the isolates. The CAAT box was absent from all but three sequences (Fig. 3). A 10 nt insertion at nt position 33 in the consensus sequence of the U3 region was identified in isolates L-11 and N-214 (Fig. 3). This insertion was also present in several published sequences, such as FESC-752, while in other sequences (M21924) it was absent. Multiple point deletions/insertions were scattered throughout the U3 region in many of the isolates (Fig. 3).

Genetic drift in passaged caprine arthritis–encephalitis viruses

To assess the in vitro stability of promoter-based TBS in milk-derived viral isolates, milk-derived cells were first cultured alone, then co-cultured with GSM cells (passage 1). Passage 1 was followed by transfer of supernatant to naive GSM cells (passage 2). Milk-derived cells from goats L-11, S-191 and N-214 were cultured in media with 1% or 2% antibiotics (Fig. 2a). After 14 days co-culture of milk-derived cells and GSM (passage 1), no amplicons were obtained by PCR of DNA from the 1% antibiotic-treated milk cells derived from any of the goats. However, in DNA samples from