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The presence or absence of the gamma-activated site determines IFN gamma-mediated transcriptional activation in CAEV promoters cloned from the mammary gland and joint synovium of a single CAEV-infected goat

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ABSTRACT

The caprine arthritis encephalitis virus (CAEV) long terminal repeat promoter was cloned and sequenced from mammary gland and carpal joint synovium isolated from a 15.5 year old, CAEV-infected Toggenburg doe with chronic mastitis and carpal arthritis. A deletion of the CAEV gamma activated site (GAS) was identified in the mammary gland but not the synovial isolate. Subsequent promoter-reporter gene construct experiments indicated that the GAS is necessary for interferon γ-mediated promoter activation. Utilizing a molecular clone of the classic isolate CAEV-CO, these findings were corroborated by a set of GAS mutant promoter–reporter constructs with and without the CAEV GAS. Results of experiments with U937 monocyte cell lines stably transfected with molecular clones of CAEV-CO GAS deletion mutants also indicated the GAS is necessary for IFNγ-mediated promoter activation. The mammary gland CAE viral isolate was propagated in caprine peripheral blood mononuclear cells and was assigned the name CAEV-MA. This is the first report describing two CAE viral isolates cloned from different anatomical locations in the same animal with and without the CAEV GAS, and is the first report detailing cytokine-induced CAEV promoter function in a naturally occurring ΔGAS promoter.

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1. Introduction

Caprine arthritis encephalitis virus (CAEV) is a lentivirus in the family Retroviridae that infects goats and is closely related to maedi-visna virus (MVV) of sheep (Benavides et al., 2009; Fields et al., 2001). CAEV infection results in two well-recognized progressive disease syndromes, encephalomyelitis in 2–4 month old goat kids and caprine arthritis in goats 12 months of age onward (Kennedy-Stoskopf et al., 1987; Maxie and Jubb, 2007). CAEV infection can also result in interstitial pneumonia and indurative mastitis, lesions similar to those observed in sheep infected with MVV (Maclachlan et al., 2011). Perhaps as a result of selection bias, CAEV-induced mastitis is less often identified and reported than lesions affecting synovial joints and the nervous system (Murphy et al., 2010). CAE-induced mastitis is a progressive clinical syndrome known as "hard udder", characterized grossly by diffuse to nodular hardening of the glands and histologically by interstitial mononuclear infiltration with lymphoid follicle hyperplasia and fibrosis (Cheevers and McGuire, 1988; Lerondelle et al., 1989; Milhau et al., 2005).

CAEV is primarily transmitted from dam to kid via colostrum, although horizontal transmission between adults is known to occur, likely as a result of respiratory secretions. In natural infections, viral expression regresses with mammary involution and reactivates at subsequent mammogenesis, such that peak viral production corresponds to the most favorable time for transmission (Clements and Zink, 1996). Although joint synovium and the central nervous system are better studied target organs of CAEV, the mechanism of goat to goat transmission suggests that the mammary gland may be a more important target organ from an epidemiological standpoint (Kennedy-Stoskopf et al., 1985).

After almost four decades of study, the mechanism of CAEV-induced tissue pathology is still incompletely understood. However, *in vivo* and *in vitro* experiments indicate that proinflammatory cytokines play an important role in lesion development (Murphy et al., 2006). The CAEV promoter is responsible for regulating viral transcription and viral transcription is thought to be partially regulated and dependent upon the presence of intralesional cytokines. It is known that high CAE viral titers correlate with lesion severity (Cheevers et al., 1988, 1991; Fluri et al., 2006; Ravazzolo et al., 2006). It seems reasonable, therefore, that factors that augment CAEV transcription play a role in lesion

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pathogenesis (Murphy et al., 2007). CAEV transcription is regulated by the viral promoter, which resides in the U3 region of the viral long terminal repeat. The cytokine interferon gamma (IFNγ) has been previously demonstrated to be a potent activator of the CAEV promoter (approximately 28-fold) through mechanisms involving the U3 region (Murphy et al., 2007; Sepp and Tong-Starksen, 1997; Tong-Starksen et al., 1996). IFNγ-activation of CAE viral transcription is mediated through the STAT1 pathway and requires the gamma activated site (GAS) within the CAEV promoter (Sepp and Tong-Starksen, 1997).

In a study characterizing multiple wild-type CAE viral promoter regions isolated and sequenced from 24 naturally infected goats, the GAS was found to be absolutely conserved in 40 of 41 cloned viral promoter sequences, suggesting that the motif is important for viral pathogenesis (Murphy et al., 2010). In the current study, we characterize a wild-type CAEV promoter and virus isolated from a naturally infected caprine mammary gland with a disrupted GAS (ΔGAS) in the viral promoter. A second CAEV promoter possessing a consensus copy of the viral GAS was cloned from the carpal joint synovium of the same goat. We hypothesized that the presence or absence of the promoter GAS in these two viral isolates determines transcriptional responsiveness to IFNγ. Objectives for this study included (i) cloning and sequencing the viral promoters, (ii) isolating and propagating mammary gland-derived virus, (iii) comparing the transcriptional function of the cloned promoters in an *in vitro* reporter gene assay, and (iv) contrasting these results with GAS-mutants derived from a molecular clone of CAEV-CO. Here we found that the CAEV GAS in the context of an intact, wild-type promoter was required for IFNγ-induced transcriptional activation. These results were corroborated by a set of promoter mutagenesis experiments using the classic isolate CAEV-CO. A more detailed understanding of the mechanisms of CAEV promoter regulation may be important as these mechanisms likely play a role in both CAE viral persistence and transmission.

2. Materials and methods

2.1. Animals and pathology

A 15.5 year old Toggenburg doe with chronic mastitis and arthritis (goat A) was humanely euthanized and a complete necropsy was performed at California Animal Health and Food Safety Laboratory, Davis. The goat was last fresh at 13 years of age and had tested serologically positive for CAEV antibody at multiple time points during life and immediately prior to euthanasia (CAEV antibody test kit cELISA, VMRD, Pullman, WA). Mammary gland tissue was aseptically collected for both *ex vivo* explant culture and DNA isolation. A complete set of tissues was collected and immediately saved in 10% buffered formalin. Formalin-fixed tissues were trimmed after 48 h fixation, embedded in paraffin and routinely processed for histological examination. Caprine tissues were examined by a board certified veterinary pathologist (BM).

A 12 year old Toggenburg doe (goat B) was co-housed with goat A from 3 years of age (2002) until goat A was euthanized (December 2010). Goat B tested seronegative for CAEV by ELISA at the time of introduction to goat A and seroconverted to CAEV approximately 2 years later (presumed horizontal transmission). Goat B's only contact with goats other than A was a hand mating to a CAEV positive buck in 2007 (no oral/nasal contact was allowed). Goat B had a chronic history of caprine arthritis and mastitis. At 12 years of age, a fresh milk sample was obtained from goat B and processed as described below (milk processing). Approximately 6 months later, goat B was euthanized; a full necropsy and tissue collection was performed.

2.2. Mammary gland explant co-culture and viral propagation

During the necropsy procedure of goat A, an approximately 3 cm^3 section of mammary gland was aseptically collected, minced using sterile equipment and placed in a 10 cm tissue culture plate. The explanted mammary tissue was cultured in Dulbecco's Modified Eagle Media (DMEM, Hyclone) with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Hyclone), 0.1 μg phorbol myristate acetate (PMA, Sigma–Aldrich) ml^{-1} and 5 μg Concanavalin A (MP Biomedicals, LLC) ml^{-1} for 2 days at 37 °C, 5% CO_2. After two days, 5 ml of the explanted mammary gland culture media was inoculated onto confluent goat synovial membrane cells (GSM). GSM cells were derived from fetal goat tissues as previously described (Klevjer-Anderson and Cheevers, 1981). The balance of the mammary gland explant culture media was clarified by centrifugation, aliquoted and stored at −80° C. The media was exchanged for fresh media at 2 and 7 days of incubation. After 14 days of incubation, the GSM cells were fixed with methanol and stained with crystal violet.

Viral propagation and determination of tissue culture infectious $dose_{50}$ ($TCID_{50}$)/ml) was initially attempted in GSM cells as previously described (Klevjer-Anderson and Cheevers, 1981). The mammary gland-derived CAEV isolate was assigned the name CAEV-MA, after the mammary tissue from which it was isolated. In addition to GSM cells, a variety of other ruminant cell lines were utilized in an attempt to propagate CAEV-MA. These cell lines included ovine fetal tracheal cells, bovine turbinate cells and ovine microglial cells. All of the cell lines were gifts from Dr. J. Stanton, Washington State University, except for the ovine microglial cells, which were provided by Dr. T. Baszler, Washington State University. Cells were plated in a 6 well tissue culture plate in DMEM (Hyclone) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Hyclone), allowed to achieve ~70% confluency and infected with 100 μl of clarified supernatant from minced mammary gland cultures (cultured for 2 days *ex vivo*). Cells were incubated for 14 days at 37 °C with 5% CO_2, the media was harvested and attached cells were stained with crystal violet to identify the presence of cytopathic effect (*e.g.* multinucleate syncytia) (Klevjer-Anderson and Cheevers, 1981). Positive control wells consisted of GSM cells infected with a known titer of CAEV-CO virus while negative control wells were treated with sterile media alone.

In addition to the ruminant cell lines, fresh EDTA-treated whole caprine blood was obtained by jugular venipuncture from a CAEV negative goat and peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation through Ficoll-Hypaque (Sigma–Aldrich, St. Louis, MO). PBMC were cultivated using previously described protocols (Sparger et al., 1994) with the modification that cells were also cultured with 0.1 μg PMA (Sigma–Aldrich) ml^{-1} in addition to 5 μg Concanavalin A (ThermoFisher Scientific) ml^{-1} for activation of viral gene expression. PBMC cultures were provided with fresh media at day 5 and were infected with 1 ml of clarified supernatant from goat A minced mammary gland cultures (cultured 2 days *ex vivo*). The media was changed every 2 days for a total of 13 days. *Ex vivo*-infected PBMC were processed for nucleic acid and assayed by PCR as described below.

2.3. Mammary gland nucleic acid isolation, cloning and sequencing

Goat A tissue DNA was isolated from 2 μm × 25 μm thick sections of formalin-fixed paraffin scrolls isolated from both caprine mammary gland and joint synovium (QIAamp DNA FFPE Tissue Kit, Qiagen). The CAEV LTR region was amplified via standard PCR using the Rev_{for} primer (5′-GGA GAT CTG AGT TCT AGG AGA GTC CCT CC) and the R_{rev} primer (5′-AGT GGA TCC TGC GAG AGC CGC TCT G)

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