Localization of a TNF-activated transcription site and interactions with the gamma activated site within the CAEV U3 70 base pair repeat

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Abstract

The cytokines TNFα and IFNγ have previously been shown to activate caprine arthritis encephalitis virus (CAEV) transcription. Increased viral titers correlate with increased lesion severity. Therefore, TNFα and IFNγ may augment the caprine arthritis lesion by increasing viral titers. CAEV transcription is under the control of the viral promoter within the U3 region of the long terminal repeat. A set of U3 deletion mutants was generated and used to establish stably integrated, U937-based cell lines. These cell lines were utilized to define the required promoter sequences for cytokine-induced transcriptional activation. Here we have identified a novel 17 nucleotide TNF-activated site within the U3 region 70 bp repeat which is both required and sufficient in a minimal construct for TNFα-induced CAEV transcriptional activation. In contrast to the results of previous studies with IFNγ, we found that multiple sequences within the U3 region 70 bp repeat were required for IFNγ-activation of the CAEV promoter. The results identify previously unrecognized complexity in the CAEV promoter that may be relevant to viral replication and disease. Published by Elsevier Inc.

Keywords: CAEV; LTR; SRLV promoter; TNF alpha; IFN gamma

Introduction

Caprine arthritis encephalitis virus (CAEV) and the ovine maedi visna virus (MVV) are closely related small ruminant lentiviruses (SRLV) that infect goats and sheep (Narayan et al., 1980). The SRLVs are monocyte–macrophage–tropic viruses which can induce persistent systemic diseases affecting the synovial joints, mammary gland, lung and central nervous system after long incubation periods (Kennedy-Stoskopf et al., 1987) The principal clinical manifestation of CAEV infection in goats is caprine progressive arthritis (CPA), a chronic synovitis characterized by excessive synovial fluid accumulation, mononuclear cell infiltration of the synovium, periarticular swelling, soft tissue mineralization and erosion of articular surfaces (Wilkerson et al., 1995).

The mechanisms of CAEV pathogenesis remain controversial, in particular mechanisms that continually promote inflammation in persistent infections are incompletely understood. Previous studies with SRLV have indicated that viral persistence is maintained by restricted viral gene expression (Gendelman et al., 1985; Peluso et al., 1985) resulting in latently infected cells that are invulnerable to immunologic control (Cheevers et al., 1988). However, the temporal regulation of SRLV gene expression and the interdependent relationship of virus and host cell gene regulation remains poorly understood. It is known that a high CAE viral titer correlates with the development of arthritis lesion severity (Cheevers et al., 1988, 1991; Fluri et al., 2006; Ravazzolo et al., 2006). Factors that augment CAE viral titers therefore likely play a role in CPA pathogenesis. CAEV transcription depends on the viral promoter, which resides in the U3 region of the long terminal repeat. Within the U3 region, CAEV-CO (Cork isolate) has two identical 70 nucleotide regions arranged as direct repeats, referred to as 70 bp repeats. Regions homologous to the 70 bp repeat are present in other CAEV isolates. The cytokines

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tumor necrosis factor alpha (TNFα) (Murphy et al., 2006) and interferon gamma (IFNγ) (Sepp and Tong-Starksen, 1997; Tong-Starksen et al., 1996) have been demonstrated to activate the CAEV promoter through mechanisms involving the CAEV U3 region.

TNFα is considered to be a key cytokine promoting inflammation in the human disease rheumatoid arthritis (RA) (Lechner et al., 1996). In the CPA lesion, the pattern of cytokine expression is similar to that found in RA (Lechner et al., 1997). Numerous experimental observations support an association of TNFα expression and CAEV infection. TNFα mRNA has been detected in the joint synovium as early as 6 days after infection with CAEV (Lechner et al., 1997) and CAEV infected goats have elevated serum TNFα levels when compared to uninfected control goats (Mduvwwa et al., 1994). LPS-stimulated monocytes from CAEV infected goats produce 200% more TNFα activity relative to uninfected goat monocytes (Werling et al., 1994). Most importantly, macrophages producing abundant TNFα mRNA have been demonstrated in synovial tissue of goats with CPA lesions and correlate with lesion severity (Lechner et al., 1996, 1997). Therefore, defining the role of TNFα regulation of CAEV transcription seems critical to a more complete understanding of CAEV pathogenesis.

Although mononuclear cells expressing IFNγ mRNA have been identified within the CPA lesion, these cells are in the minority relative to TNFα-expressing cells (Lechner et al., 1997). Nevertheless, double staining experiments of CPA lesions have revealed that some of the cells expressing viral RNA strongly express MHC II molecules and that IFNγ-expressing cells co-localize with areas of detectable CAEV expression (Lechner et al., 1997). Importantly, macrophage activation and MHC II expression is associated with exposure to IFNγ or TNFα (Mosser, 2003). These observations suggest the possibility that local CAEV expression is induced in part by local production of cytokines and that TNFα may be more important than IFNγ within the chronic CPA lesion.

IFNγ-activation of CAE viral transcription is mediated through the STAT1 pathway and requires the gamma activated site (GAS) within the U3 region 70 bp repeat (Sepp and Tong-Starksen, 1997). However, the dependence of IFNγ induction on other sequences within the CAEV 70 bp repeat has not been explored and less is known about the mechanism of TNFα in regulating CAEV expression. The Fos-Jun heterodimer AP-1 has been shown to be required for MVV transcription (Shih et al., 1992). We recently demonstrated that TNFα-induced activation of CAEV transcription is independent of AP-1 (Murphy et al., 2006). Consequently, other sequences in the U3 promoter must regulate TNFα-induced expression. A more detailed understanding of cytokine-induced signaling mechanisms underlying CAEV transcriptional activation may be important as these mechanisms likely play a role in viral persistence. Our results identify a TNF-activated site in the U3 region 70 bp repeat that functions autonomously to IFNγ regulatory elements. Surprisingly, the GAS previously identified as an IFNγ response element (Sepp and Tong-Starksen, 1997) did not function independently within the U3 region 70 bp repeat. Results presented here clarify some regulatory elements that exist in the CAEV promoter and indicate the existence of additional promoter complexity.

Results

IFNγ and TNFα activate CAEV expression in U937\textsubscript{CAEV} cells

To confirm that IFNγ activates CAEV transcription in U937\textsubscript{CAEV} cells, the cells were exposed to various concentrations of recombinant human IFNγ for varying lengths of time. Gag mRNA copy number was determined by real time RT-PCR. U937\textsubscript{CAEV} cells exposed to 400 U IFNγ ml\textsuperscript{−1} of media for 0–48 h had a peak Gag mRNA expression at 4 h (Fig. 1a). The concentration of 400 U IFNγ ml\textsuperscript{−1} has previously been demonstrated to be the optimal concentration for IFNγ induction of the CAEV promoter (Tong-Starksen et al., 1996). U937\textsubscript{CAEV} cells exposed to 0–400 U IFNγ ml\textsuperscript{−1} of media for 4 h had a dose–response activation of CAEV Gag mRNA expression with a maximal activation at 400 U ml\textsuperscript{−1} of media (Fig. 1b). These experiments were each repeated twice with similar results. Treatment of U937\textsubscript{CAEV} cells with either TNFα or IFNγ or both cytokines together (10 ng or 400 U ml\textsuperscript{−1} media, respectively) for 4 h activated CAEV transcription (p < 0.05, Fig. 1c). The average fold-activation for TNFα or IFNγ treated cells was 3.4- and 28-fold, respectively. Treatment of U937\textsubscript{CAEV} cells with both TNFα and IFNγ (10 ng and 400 U ml\textsuperscript{−1} media, respectively) did not result in significant activation beyond that for IFNγ alone (p > 0.05, Fig. 1c). An additive or synergistic effect for cells treated with both cytokines was therefore not identified. This experiment was repeated three times with similar results. Samples without reverse transcriptase (RT−) did not yield Gag amplification products in any of these experiments, indicating that the DNase treatment step was effective. These results demonstrate differential activation of the CAEV promoter by TNFα and IFNγ in the U937\textsubscript{CAEV} system and are consistent with prior published results (Sepp and Tong-Starksen, 1997; Tong-Starksen et al., 1996).

The U3 70 bp repeat is required for both TNFα and IFNγ-activated activation of the CAEV promoter

In order to define promoter elements that regulate cytokine-induced transcriptional activation, two U937 cell lines were generated with deletions in the CAEV U3 region, U937\textsubscript{CAE\textsubscript{Δ1}} and U937\textsubscript{CAE\textsubscript{Δ2}} (Fig. 2a), as described previously (Murphy et al., 2006). The U937\textsubscript{CAE\textsubscript{Δ1}} and U937\textsubscript{CAE\textsubscript{Δ2}} cell lines were treated with or without 10 ng TNFα ml\textsuperscript{−1} or 400 U IFNγ ml\textsuperscript{−1} for 4 h prior to RNA harvest. The Gag mRNA copy number was determined by real time RT-PCR. When corrected for equal numbers of integrating constructs, cytokine treatment of U937\textsubscript{CAE\textsubscript{Δ2}} cells resulted in transcriptional activation (p < 0.05, Fig. 2b), while cytokine-induced activation was abrogated in the U937\textsubscript{CAE\textsubscript{Δ1}} cells (§, Fig. 2c). The basal promoter function in both mutant cell lines was attenuated relative to the U937\textsubscript{CAE} cells (p < 0.05). In these experiments, samples without reverse transcriptase (RT−) did not yield Gag
amplification products. Each of these experiments was repeated four times with similar results.

The only difference between the U937 CAEΔ2 and the U937CAEΔ1 cells is the presence of a single copy of the 70 bp repeat in the U937CAEΔ2 cells. The loss of this repeat in the U937CAEΔ1 cells coincides with the loss of cytokine induction. Therefore, these results suggest that TNFα and IFNγ-induced activation of the CAEV promoter depends on the CAEV U3 70 bp repeat.

The CAEV GAS element is necessary for IFNγ but not TNFα-induced activation of the CAEV promoter

IFNγ activates Stat-1 phosphorylation (Krauss, 2003), and homodimers of this transcription factor are known to bind the CAEV gamma activated site (GAS) (Sepp and Tong-Starksen, 1997). A deletion mutant based on CAEΔ2, but lacking the U3 GAS element (CAEΔGAS), was generated to examine cytokine-GAS dependence (Fig. 3a). The CAEΔGAS construct has a single copy of the 70 bp repeat with a 9 nucleotide deletion of the single remaining GAS site. A stably integrated, U937-based cell line was generated from the CAEV mutant construct as described in the materials and methods. The U937CAEΔ2 and U937CAEΔGAS cell lines were treated with or without 10 ng TNFα or 400 U IFNγ ml⁻¹ of media for 4 h prior to RNA harvest. When corrected for equal numbers of integrating constructs, the U937CAEΔGAS cells had a significant level of TNFα-induced activation (*, p < 0.05), which was similar to the level of TNFα-induced activation in U937 CAEΔ2 cells. In contrast, IFNγ-induced activation was eliminated in U937CAEΔGAS cells (§, Fig. 3b). These results indicate that CAEV promoter elements regulating induction by IFNγ or TNFα are separable. This experiment was repeated three times with similar results.

Treatment of monocytes with IFNγ induces the aggregation of the receptor complex. Subsequent recruitment of Janus kinase JAK1 and JAK2 results in the reversible phosphorylation of the receptor, creating a docking site for the src homology 2 domain of Stat-1 (Greenlund et al., 1995). Stat-1 becomes tyrosine phosphorylated, dissociates from the receptor, dimerizes and translocates to the nucleus where it binds the GAS in the promoters of IFNγ-inducible genes and initiates transcription.

To explore cytokine-induced phosphorylation of Stat-1, a western blot was performed with U937CAEV cells stimulated with or without TNFα, GM-CSF or IFNγ and evaluated for phosphorylated STAT-1. Equivalent amounts of protein were loaded per lane (50 ug) on two gels run in parallel. A band corresponding to phosphorylated Stat-1 (90 kDa) was identified in the IFNγ-treated samples but not in the other samples (Fig. 3c). The control blot incubated with anti-Stat-1 confirmed that Stat-1 protein was present in all of the treatment groups (Fig. 3d). This experiment was repeated once with similar results.

These results demonstrate Stat-1 phosphorylation in IFNγ-treated U937CAEV cells and a lack of Stat-1 phosphorylation in cells that were untreated or treated with TNFα or GM-CSF. The results are consistent with previous findings demonstrating involvement of the Stat-1 pathway and the U3 GAS in IFNγ activation of the CAEV promoter (Sepp and Tong-Starksen, 1997; Tong-Starksen et al., 1996). The results also support the concept that TNFα activates CAEV expression by a promoter element distinct from GAS.
The element required for TNFα-induced CAEV promoter activation resides in a 17 bp sequence

Since the 70 bp repeat is required for both TNFα and IFNγ-induced activation of the CAEV transcription, progressive sub-deletions of the 70 bp repeat were generated to identify important sequences. Four deletion mutants of the 70 bp repeat were generated from the CAEΔ2 parent construct, as described in the materials and methods. These deletion mutants, referred to as CAEΔA, CAEΔB, CAEΔC and CAEΔD, are contiguous 17–20 bp deletions from the 5′ end of the 70 bp repeat (Fig. 4a). Stably integrated, U937-based cell lines were generated from each CAEV-mutant construct as described in the materials and methods. A PCR reaction was performed with genomic DNA from each cell line and the Revfor and Rrev primer set. The resulting PCR restriction products matched the predicted PCR product sizes for each deletion mutant (data not shown).

Each of the mutant cell lines were treated with or without 10 ng TNFα ml⁻¹ (Figs. 4b and c) or 400 U IFNγ ml⁻¹ (Fig. 4d) for 4 h prior to RNA harvest. The Gag mRNA copy number was determined by real time RT-PCR. When corrected for equal numbers of integrating constructs, TNFα-induced activation was abrogated in U937CAEΔC cells (§, Fig. 4b), whereas TNFα-induced promoter activation was present in the other 3 cell lines (*, p < 0.05, Figs. 4b and c). Each of these experiments was repeated three times with similar results. In these experiments, samples without reverse transcriptase (RT−) did not yield gag amplification products.

These results suggest that the putative TNFα-activated site or sites resides within the 17 nucleotide sequence deleted in the CAEΔC construct: 5′′AAGAAAAGCAAGTTCAC. This 17 nucleotide region, identified here as the TNF-activated site (TAS), is apparently required for TNFα-induced promoter activation. Two additional deletion constructs were generated to divide this region into 5′′ and 3′′ subregions. The constructs CAEΔE and CAEΔF were generated from the parent construct CAEΔ2 and have contiguous block deletions of either the 5′′ end (5′′AAGAAAAGC) or the 3′′ end (5′′AGTTC), respectively. These constructs were utilized to generate two U937-based cell lines, U937CAEΔE and U937CAEΔF. TNFα-activation was abrogated in the U937CAEΔE cell line and was attenuated in the U937CAEΔF cell line relative to U937CAEΔ2 cells (data not shown). The 5′′ AAGAAAAGC sequence within locus C is therefore apparently required while the 3′′ AGTTC sequence...
may be important for full TNF-α-induced activation. The result of experiments with the U937CAEΔE cell line therefore independently confirms the negative result of the U937CAEΔC cell line.

In contrast to the effect identified with TNFα, IFNγ-induced promoter activation was abrogated in each of the 70 bp sub-deletion mutants (§, *p > 0.05, Fig. 4d). The lack of IFNγ-induction in the U937CAEΔA cell line is consistent with results in which the GAS was deleted (U937CAEΔGAS, Fig. 3). However, the lack of IFNγ-activation in the U937CAEΔB, U937CAEΔC, and U937CAEΔD cell lines is an unexpected and interesting finding. A previous study showed that a single GAS placed upstream from a herpes simplex virus thymidine kinase (tk) minimal promoter and a chloramphenicol acetyl transferase (CAT) reporter gene was sufficient to activate transcription (Sepp and Tong-Starksen, 1997).

The TNF activated site is sufficient for TNFα-induced activation of a CAEV minimal promoter

To determine if the 17 nucleotide TAS is sufficient to confer TNFα-induced activation, two deletion constructs were generated from CAEΔ2, CAEΔ1 and CAEΔJ (Fig. 5a). Both of these mutants lack the A, B and D regions of the 70 bp repeat. The CAEΔ1 mutant has the 5′ portion of the U3 region placed adjacent to TAS, while CAEΔJ lacks the 5′ U3 region. The sizes of the constructs were confirmed by PCR analysis of genomic DNA. The PCR products generated from U937CAEΔJ and U937CAEΔI genomic DNA were sequenced and determined to be identical to the predicted sequences. Promoter activation was induced by TNFα in the U937CAEΔ2 and U937CAEΔJ cells (*, *p < 0.05, Fig. 5c). In contrast, TNFα-induced activation was not detected in U937CAEΔI cells (§, *p > 0.05), indicating a lack of TNFα responsiveness when the 5′ portion of the U3 region was placed adjacent to TAS. These experiments were repeated three times with similar results.

Summary of the CAEV U3 mutant basal promoter function and cytokine induced activation

The CAEV-CO U3 promoter region and each of the derived deletion mutants are represented schematically in Fig. 6. The mean fold-activation by cytokine and the basal promoter
activities for each mutant are listed. The mean fold activation level for TNFα and IFNγ are 3.4 and 28 fold, respectively. The basal promoter activities are attenuated for the CAEΔ1 and CAEΔ2 constructs relative to the CAEV-CO, suggesting that the 70 bp repeat and sequences between 8998 (the 3′ end of the 70 bp repeat) and 9072 (the TATA box) contribute to CAEV basal promoter function.

Sequence conservation within the of the U3 promoter across multiple isolates of CAEV

The GAS and TAS appear to be important for cytokine-induced activation of the CAEV-CO promoter. In addition, regions B, C and D appear to be required for IFNγ-induced CAEV expression. To assess sequence properties responsible for these characteristics, the analogous promoter regions of three other CAEV isolates (CAEV-1g5, -63 and -gansu) were analyzed to determine the level of sequence conservation. In these isolates, the GAS and TAS had a 90.9% and 88.2% sequence conservation, respectively (Fig. 7). By contrast, the overall sequence conservation for the 70 bp repeat and the entire U3 region were 78.9% and 50.9%, respectively. In the D region, sequence conservation was 85%, and it was 82.4% for region B. The B region conserved nucleotide distance between GAS and TAS among the four CAEV isolates and this sequence was biased towards G and T residues. Consequently, multiple sequence regions that contribute to cytokine-induced activation conserve spacing and/or nucleotide sequence.

Discussion

Previous experiments exploring cytokine-induced activation of the CAEV promoter were performed with U3-reporter gene constructs in episomal transient transfection assays (Sepp and
Fig. 5. Region C is sufficient for TNFα-induced activation of a CAEV minimal promoter. (a) Maps of deletion mutants. The U937\textsubscript{CAEΔ2} mutant has a U3 region deletion from 8923–9069. The plasmids CAEΔA and CAEΔJ mutants were derived from CAEΔD and have an additional deletion from 8856–8889 or 8819–8889, respectively. (b) A PCR reaction with genomic DNA isolated from U937\textsubscript{CAEΔ2}, U937\textsubscript{CAEΔA}, U937\textsubscript{CAEΔJ} and U937\textsubscript{CAEV} cells was performed with the Rev\textsubscript{rev} and R\textsubscript{rev} primer set; the PCR products were electrophoresed on an agarose gel (lanes 1–4, respectively). (c) Real time RT-PCR analysis for Gag mRNA copy number (normalized) in U937\textsubscript{CAEΔ2}, U937\textsubscript{CAEΔA} and U937\textsubscript{CAEΔJ} cells with exposure to 10 ng TNFα ml\textsuperscript{-1} media (light grey bars) or no treatment (black bars) for 4 h. Relative to untreated cells, TNFα-induced activation was identified for U937\textsubscript{CAEΔ2} and U937\textsubscript{CAEΔJ} cells (*, p < 0.05). TNFα-induced activation was not identified for U937\textsubscript{CAEΔA} cells (§). The RT-lane corresponds to untreated control sample lacking reverse transcriptase.

Tong-Starksen, 1997; Tong-Starksen et al., 1996). The experiments described here and elsewhere (Murphy et al., 2006) utilized monocyte cell lines generated with stably integrated, CAE proviral constructs and quantitatively assessed viral Gag mRNA copy number as a measure of the CAEV promoter activity. Hence, these constructs are intended to more closely replicate the natural host cell–viral interactions. Although U937 is a human-derived cell line, previous experiments with stably integrated CAEV constructs have demonstrated cytokine activation effects in both U937 cells and goat synovial membrane cells (Murphy et al., 2006).

The most important finding presented here is the delineation of multiple regulatory elements in the CAEV U3 promoter. These regulatory elements appear to function by promoting viral transcription on exposure of host cells to the cytokines TNFα or IFNγ. Transcriptional regulation by this promoter region is more complex than previously envisioned. It was proposed that the AP-1 factor activates CAEV transcription by binding AP-1-like sequences within the CAEV viral promoter (Kalinski et al., 1994). However, TNFα and GM-CSF activation of the CAEV promoter was found to be independent of AP-1 (Murphy et al., 2006). Results described here demonstrate that a 17 nucleotide region within the U3 70 bp repeat, the TNF activated site (TAS), is required for TNFα-induced transcriptional activation. The TAS, when positioned upstream from the TATA box, was also sufficient to support TNFα induction of Gag transcription. However, full TNFα-induced activation may require cooperation of both TAS elements and possibly other as of yet unidentified LTR sequences. Furthermore, the TAS functions autonomously to the known IFNγ response element (GAS). Although the minimal sequence of the TAS was not defined, data from two additional promoter subdeletions (CAEΔE and CAEΔF) indicated that deletion of the first nine nucleotides (5′ AAGAAAAGC) abrogated the activation while deletion of five nucleotides near the 3′ end (5′ AGTTC) reduced TNFα-induced activation (data not shown). Therefore, we have identified a promoter region that appears to be important for TNFα regulation of CAEV in vitro. The transcription factor(s) and signaling mechanism mediating TNFα-induced activation of the U3 promoter remain undefined. However, the TAS demonstrates some sequence similarity to a tandem repeat of heat shock promoter elements.

In contrast to previous findings, the U3 GAS element did not function autonomously in constructs investigated here. In previous experiments, GAS positioned upstream from a tk minimal promoter demonstrated that this site was sufficient to support IFNγ-induced expression of a CAT reporter gene (Sepp and Tong-Starksen, 1997; Tong-Starksen et al., 1996). Our results using stably-integrated CAEV constructs showed that the GAS is required for IFNγ-induced activation (in both the CAEΔA and CAEΔGAS constructs). However, sequential deletion of other sequences within the 70 bp repeat downstream of GAS (CAEΔB, CAEΔC and CAEΔD) abrogated activation. These findings suggest that the GAS within the context of the isolated tk minimal promoter functions differently than promoter sequences in the context of the CAEV genome. The identification of additional sequences that contribute to IFNγ inducibility may help clarify the in vivo requirements for this response.

The abrogation of IFN-gamma inducible Gag expression by the elimination of sequence blocks B, C or D indicated a regulatory interaction not previously recognized for the CAEV promoter. Several alternative explanations were considered for
possible roles of these sequences in this interaction: i) these sequences blocks provide required spacing separating the GAS from the TATA box, but lack a specific sequence requirement; ii) they are conserved sequences that interact with other regulatory factors necessary for IFN-\(\gamma\) inducibility; or iii) they provide required spacing, but are also conserved sequences that interact with regulatory factors independent of IFN-\(\gamma\) inducibility (as may be the case with sequence block C, or TAS). Promoter sequences of four CAEV isolates showed that the location and spacing of GAS and TAS were conserved within the 70 bp repeat. However, in addition to distance, sequences in each block (A–D) were highly conserved among the isolates (Fig. 7). Although some variability was observed in sequence block B, this sequence was characterized by a bias towards G and T nucleotides (12 of 17 nucleotides). Consequently, while these sequence blocks may have a spacer function, the high degree of nucleotide conservation also suggests roles that supercede a simple spacer function. While conserved sequences in B and D could be candidates for possibilities ii) or iii), our data do not allow us to distinguish which is more likely. Essential functions of the 70 bp repeat may remain to be clarified.
Another complexity observed in our results was that the 37 nucleotides of the 5′-most U3 promoter sequence upstream of the 70 bp repeat might have a suppressor function. TNFα-induced activation was abrogated in U937CAEVΔ cells, which have the 5′ promoter region adjacent to TAS. An AP1-like sequence, TGAGACA, is present within this 5′ promoter region of all four CAEV isolates. This sequence may provide a binding site for AP1 factors, and may function as a negative regulatory element in CAEV transcription. In support of this possibility, previous results demonstrated CAEV transcriptional activation in response to treatment with a pharmacologic inhibitor of AP-1 (Murphy et al., 2006). While it is possible that this region has a suppressor function, it is also possible that this effect is an artifact of the construct itself. Additional research is needed to distinguish these possibilities.

A final consideration is the effect of promoter deletion on the basal promoter function. The basal function of the promoter was found to be affected by deletions of sequences between the 70 bp repeat to the TATA box and as of yet undefined sequences within the 70 bp repeat (Fig. 6). These conclusions concerning the importance of specific sequences for basal promoter function are essentially the same as for previously published data (Murphy et al., 2006).

Collectively, these data provide significant resolution to an emerging model of the CAEV promoter region. The U3 70 bp repeat is a core promoter element and is conserved in multiple CAEV isolates. The 70 bp repeat contains at least two distinct regulatory elements, the GAS and TAS. Delineation of these two regions identified adjacent regions (B and D) that conserve spacing and nucleotide sequence among four different CAEV isolates. In addition, a sequence 5′ to the 70 bp repeat resembles an AP-1 binding site, which warrants investigation as a repressor element. Since, some isolates contain two 70 bp repeats, additional complexity in this promoter region might be expected. The organization presented here provides specific guidance for further delineating regulatory elements that mediate CAEV replication and gene expression. These considerations may be important because previous experiments suggested that CAEV isolates with different promoter sequences vary in virulence (Cheever et al., 1988). While obtained from a human cell line, our results identify sequences that can now be investigated to determine connections between the CAEV promoter sequences and the caprine progressive arthritis lesion.

CAEV promoter complexity may be relevant to variable cytokine environments encountered by the virus within the host. For instance, IFNγ expressing lymphocytes are relatively common in subscapular lymph nodes draining severely affected joints (Lechner et al., 1997). In addition, IFNγ expression colocalizes with viral RNA expression, suggesting the possibility that this cytokine activates viral expression in adjacent macrophages. In other studies, lymph nodes from CAEV infected animals have been identified as important viral reservoirs (Ravazzolo et al., 2006). These observations suggest that IFNγ may be important in maintaining CAEV expression in the lymph nodes of chronically infected goats. However, within the chronically inflamed synovial joint lesion, more cells were detected that express TNFα than IFNγ mRNA (Lechner et al., 1997). Under these circumstances, it is possible that TNFα represents the dominant regulatory cytokine for CAEV replication. Therefore the CAEV promoter region may be adapted to respond to different cytokine environments that vary among different host tissues. There is precedence for local cytokine environments differentially regulating viral transcripts in macaques infected with the simian immunodeficiency lentivirus (Orandle et al., 2001). With the identification of sequence elements that regulate IFNγ and TNFα responses of CAEV, it becomes feasible to investigate these possibilities. Differences observed in the strength of activation by IFNγ (28-fold) and TNFα (3.4-fold) may also be relevant to in vivo CAEV interactions with the host and require further investigation.

Materials and methods

Plasmids

The plasmid CAEVpac11 was generated by Dr. Isidro Hötzel as described previously (Murphy et al., 2006). This plasmid has the CAEV-CO strain (Cork and Narayan, 1980) proviral sequence with a 1330 bp deletion in env and an SV40-pac (pac: puromycin acetyltransferase) cassette replacing the tat gene. The plasmids CAEVΔ1 and CAEVΔ2 were derived from CAEVpac11 and have a deletion between nucleotides 8856–9069 or 8923–9069 of the CAEV-CO promoter region, respectively. The plasmids CAEVΔ1 and CAEVΔ2 were generated as described previously (Murphy et al., 2006). The plasmids CAEVΔGAS, CAEVΔA, CAEVΔB, CAEVΔC and CAEVΔD are derived from CAEVΔ2 and have additional block deletions between nucleotides 8863–8872, 8856–8872, 8873–8889, 8890–8906 and 8907–8926, respectively. For each deletion mutant, 5′ and 3′ primer pairs were utilized in PCR reactions to generate 5′ and 3′ PCR products with unique restriction sites at both ends. For the CAEVΔGAS construct, the 5′ primer pair was Revfor and GASrev while the 3′ primer pair was Rrev and GASfor. The 5′ and 3′ primer pairs for the other constructs were as follows—CAEVΔA: Revfor/ΔArev and Brev/GASfor; CAEVΔB: Revfor/ΔBrev and Rrev/ΔBfor; CAEVΔC: Revfor/ΔCrev and Rrev/ΔCfor and CAEVΔD: Revfor/ΔDrev and Rrev/ΔDfor. The two PCR products for each construct were ligated together and subsequently ligated into the pCAEVpac11 plasmid backbone lacking the 3′ LTR.

The constructs CAEVΔ1 and CAEVΔ2 were based upon the CAEVΔD and were generated in a similar manner as CAEVΔA–CAEVΔD. They each have an additional block deletion between nucleotides 8856–8889 or 8819–8889, for CAEVΔ1 and CAEVΔ2, respectively. The 5′ and 3′ primer pairs for CAEVΔ1 were Revfor/Δ1rev2 and Rrev/Δ1for2 and CAEVΔ2 were Revfor/Δ1rev1 and Rrev/Δ1for1. The two PCR products for each construct were ligated together and subsequently ligated into the pCAEVpac11 plasmid backbone lacking the 3′ LTR.

All cloning steps involving PCR were checked by sequencing to confirm the absence of PCR-induced sequence errors. The plasmid pMEVSV-G, expressing the vesicular stomatitis...
virus (VSV) G glycoprotein (utilized for packaging), was obtained from Richard Sutton. All plasmids were propagated in Escherichia coli JM109.

Cells and tissue culture media

The U937 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). U937 cells were maintained at 37 °C in 5% CO2 in RPMI 1640 media with 10% FCS, 100 U penicillin L−1 and 2-mercaptoethanol (2 μl 500 ml−1 media). 293T cells (utilized for generating pseudotyped virus) were grown in Dulbecco’s Modified Eagle Medium (DMEM, Gibco BRL, Grand Island NY) supplemented with 10% FCS, 100 U penicillin L−1, 100 μg streptomycin L−1 and 2 mM l-glutamine. Culture medium was replaced with 8 ml RPMI 10% pseudotyped virus. Control U937 cells were placed in 4 ml of RPMI 10% tissue culture flask with 2 ml RPMI 10% 12% fetal calf serum (FCS), 100 U penicillin L−1 and stored at −20 °C. The U937 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). U937 cells were divided into 200 μl aliquots. Puromycin (Sigma Aldrich) was solubilized in PBS with 0.1% bovine serum albumin at stock concentrations of 10 μg ml−1. The cytokines were filter sterilized and split into 20 μl aliquots. Puromycin (Sigma Aldrich) was solubilized in water at a stock concentration of 2 mg ml−1, filter sterilized and divided into 200 μl aliquots. All of the reagents were protected from light and stored at −20 °C.

DNA primers and probes

Primers for RT-PCR and fluorescence-interference probes for real time PCR were ordered from Integrated DNA technologies, Inc. (IDT, Coralville, IA). Primers were designed as follows: CAEV Gagfor primer: 5′ ATT CTG TTA TGG TCC AGC AAC TGC; CAEV Gagrev primer: 5′ ATA TGC CAA CTT CTT TCC AAA GTC 3′; Revfor primer: 5′ CTC AGG ATG GGA ATC TTC GGG GCT GAG AGC CGC TCT; GASfor primer: 5′ GGG CTC GAG GAG GGG GTT ATA AGA AGA GC; GASSrev primer: 5′ CCC CTC GAG TTT GGC CTA GCT GCT TGT TAT TAG TCC TC; ΔACrev primer: 5′ CCC CTC GAG AGC TGG TTA TTA GTC CTC TCT TTC AGC CC; ΔBrev primer: 5′ GGC GTC GAG AAG AAA AGC AAG TCT ACT ATG ACA AAG C; ΔBrev primer: 5′ CCC CTC GAG TTA CAG GAA TTT GGC CTA GCT GTG TAT TAG; ΔCrev primer: 5′ GGG GTC GAG TAC AAA GCA AAA TGA GAT CTG C; ΔCrev primer: 5′ CCC CTC GAG ATA CCC CCC CAA GTG ATT TAC AGC; ΔDrev primer: 5′ GGG CCC GCT GAG AGA TGG TTA TTA GTC CTC TCT GCT GTA TAT AAG GAG AAC C; ΔDrev primer: 5′ CCC CTC GAG GTG AAC TTG CTT TCTCTA TAA CCC; ΔIfor primer: 5′ GGG CCA TGG AAG AAA AGC AAG TCT ACC TCG AGA GAT CTG C; ΔIrev1 primer: 5′ CCC CCA TGG TCA CCC CCT TTT TT TTA TTT TTG TGG TGT TCT C; ΔIrev2 primer: 5′ CCC CCA TGG AGC TTG TTT TTA TTA GTC CTC TTT AGC CC; 18s rRNAfor primer: 5′ GGA ACC GCT TGA ACC CCA TT 3′; 18s rRNArev primer: 5′ CCA TCC AAT CCG TAG TAG CG. The primers were utilized in PCR reactions at a final concentration of 400 nM.

The CAEV Gag fluorescence-interference probe for real time PCR was HPLC purified (IDT, Coralville, IA) and utilized at a final concentration of 250 nM. The Gag probe was designed as follows: 5′-5HEX/AGC AAT GCA GCA TGG CCT GTG TTC/BHQ-1/-3′ (where BHQ_1 represents Black Hole Quencher). The probe was protected from light and stored at −20 °C.

RNA isolation/RT PCR assays

Total cellular RNA was purified utilizing Trizol Reagent (Invitrogen, Carlsbad, CA). The RNA was isolated according to the protocol included with the kit and, if not utilized immediately, was stored at −80 °C. DNase treatment was accomplished with TURBO DNase (Ambion) utilizing 4 units DNase per 3 μg total RNA and incubating the reaction for 30 min at 37 °C. The DNase was inactivated by incubating the samples at 65 °C for 15 min. RNA was reverse transcribed into cDNA with the First Strand cDNA Synthesis Kit for RT-PCR (Roche, Indianapolis, IN). A control sample was run without reverse transcriptase to control for the DNase treatment. Real time PCR assays were performed with Platinum Taq DNA polymerase (Invitrogen) or iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) on a Bio-Rad iCycler detection system. CAEV Gag or 18s rRNA primer pairs were utilized for real time PCR reactions; each assay was performed in triplicate. Each experiment was performed three or more times and the

Reagents

The recombinant human cytokines TNFα, GM-CSF and IFNγ (R&D Systems, Inc., Minneapolis, MN), were solubilized in PBS with 0.1% bovine serum albumin at stock concentrations of 10 μg ml−1. The cytokines were filtered sterilized and split into 50 μl aliquots. Puromycin (Sigma Aldrich) was solubilized in water at a stock concentration of 2 mg ml−1, filter sterilized and divided into 200 μl aliquots. All of the reagents were protected from light and stored at −20 °C.
mean values for each treatment were pooled for statistical analysis. All experiments were controlled for variations in the reverse transcriptase step and in the RNA concentration by normalizing to 18 s rRNA PCR products run in parallel with the same cDNA. The normalization calculation was performed for each sample as follows: [sample copy number/sample 18s copy number/control 18s copy number]. When experiments were performed with multiple cell lines, an integration constant was generated by the following formula: genomic U937<sub>CAEV</sub> gag copy number/cell line of interest genomic gag copy number. This integration constant was then multiplied by the value generated above to arrive at the final copy number normalized by both the amount of 18 s rRNA and the relative number of integrated constructs within the cell line genome.

Standard PCR was performed with Platinum Taq DNA polymerase (Invitrogen) on a GeneAmp PCR System 9600 (Perkin Elmer). PCR products from standard RT PCR were run on 1.5% agarose gels, stained with ethidium bromide and digitally photographed with a Multisight Light Cabin (Alpha Innotech Corporation, San Leandro, CA) utilizing Alphaimager 2200 software.

**Genomic DNA isolation and PCR product sequencing**

Genomic DNA was isolated from 8 × 10<sup>5</sup> cells utilizing a Puregene genomic DNA isolation kit (Genta Systems, Minneapolis, MN). Standard PCR was performed with the Rev<sub>60</sub> and Rev<sub>40</sub> primer set. Genomic DNA real time PCR was performed with CAEV Gag primers. In each cell line, the number of Gag copies in 100 ng of genomic DNA was determined to be: 3.07, 2.10, 2.92, 1.12, 2.22, 2.18, 5.35, 5.13, 1.58 and 0.968 (×10<sup>5</sup>), for U937<sub>CAEV</sub>, U937<sub>CAEΔI</sub>, U937<sub>CAEΔΔ</sub>, U937<sub>CAEGΔ</sub>, U937<sub>CAEEΔA</sub>, U937<sub>CAEEΔB</sub>, U937<sub>CAAB</sub>, U937<sub>CAEΔ</sub>, U937<sub>CAΔΔ</sub>, U937<sub>CAΔΔ1</sub> and U937<sub>CAΔΔ2</sub> cell lines, respectively. This data was utilized to normalize the number of integrated constructs between two or more cell lines in the same experiment (see RNA isolation/RT PCR assays, above).

**Western blots and protein assays**

3 × 10<sup>6</sup> U937<sub>CAEV</sub> cells were plated in RPMI media in 6 well plates, treated with or without TNFα, GM-CSF or IFNγ (10 ng ml<sup>−1</sup>, 50 ng ml<sup>−1</sup> or 400 U ml<sup>−1</sup>, respectively) and incubated for 4 h at 37 °C. The cells were washed once with PBS and lysed with 150 µl of lysis buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium dodecyl sulfate, 0.5% deoxycholate). The lysates were kept on ice, sheared by pipetting through a 20 gauge needle and filtered through a syringe-tip filter. If not used immediately, the lysates were stored for up to 7 days at −20 °C. The cell lysate protein concentration was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL). The assays were run in triplicate with at least two different sample dilutions. For each assay, a nine sample standard curve was generated in duplicate from the kit reagents. The sample absorption was determined on a microplate reader (Titertek Multiskan MCC/340, EFLAB, Finland) at a wavelength of 540 nm. The lysates were mixed with loading buffer (0.03 M Tris (pH 6.8) containing 2% SDS, 10% glycerol, and 0.01% bromphenol blue in the presence of 100 mM dithiothreitol) (Ozyoruk et al., 2001), heated to 100 °C for 5 min and loaded onto a 4–20% gradient polyacrylamide gel (BioRad, Hercules, CA). The gel was run at 200 V for 30 min and transblotted onto nitrocellulose at 100 V for 60 min. Western blotting was performed utilizing either rabbit polyclonal anti phospho-Stat1 (Tyr 701) or rabbit polyclonal anti-Stat1 primary antibodies (PhosphoPlus Stat1 (Tyr701 Antibody Kit, #9170, Cell Signaling Technology, Beverly, MA) according to the kit protocol.

**Sequence analysis**

The CAEV-1g5 and CAEV-63 LTR regions were cloned and sequenced in our laboratory by Isidro Hötzel (accession EF194041 and EF194040, respectively). The CAEV-gansu isolate was obtained from a public database (accession AY900630). Comparison of CAEV promoter sequences was performed utilizing Vector NTI software (Invitrogen).

**Statistics**

The data are presented as the mean of three or more values (bar) with the standard deviation displayed as error bars. The pooled data (means) from three or more independent experiments was tested for violation of normality and equal variance assumptions. If the data passed these tests, an analysis of variance was performed (ANOVA). If the data violated either of the two assumptions, a Kruskal–Wallis (ranks) procedure was performed in lieu of the ANOVA. Where global differences were identified by either procedure, the Student–Newman–Keuls multiple comparison test was used for pair wise comparisons of the mean responses between treatment groups. A p value of less than 0.05 was considered to be statistically significant. All of the figures were generated in SigmaPlot and the statistics were performed with SigmaStat software (Systat Software Inc. Richmond, CA).

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**References**


